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Neurotoxicity and Neuroinflammation in the Enteric Nervous System

by Amy Peaire

This thesis is submitted as a partial fulfillment of the Ph.D. program in Neuroscience Graduate Program

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STATEMENT OF THE CONTRIBUTION OF EACH CO-AUTHOR IN EACH PUBLICATION

Within Chapter 2, Ms. Kim Wong was responsible for the initial development of the primary cell culture technique. Amy Peaire subsequently adapted this cell culture protocol for use in pathophysiological investigations, and performed the neurochemical and morphological characterization of these cultures. Electrophysiological characterization of cultured neurons, both freshly-plated and cryopreserved, was performed by Dr. Bruce Hutcheon in Dr. Mike Poulter's laboratory at the National Research Council. Preparation of cortical cell cultures was performed by Ms. Leslie Brown from Dr. Mike Poulter's laboratory.

CD-1 mice used in Chapter 3 were provided by Dr. Hymie Anisman at Carleton University. rhTNF-α injections were performed by Shawn Hayley in Dr. Anisman's laboratory. All other experimental procedures were performed by Amy Peaire.

ABSTRACT

The enteric nervous system (ENS) is the intrinsic nervous system of the gastrointestinal (GI) tract. Damage and degeneration of the ENS is a feature of some GI diseases, such as inflammatory bowel disease (IBD), and is associated with several other diseases including diabetes mellitis and Parkinson's disease. In these diseases, ENS damage can produce disordered or impaired gastrointestinal movement, nausea, vomiting, weight loss and malnutrition. To date, few specific factors have been implicated in the pathogenesis of ENS degeneration, and little research pointing to methods of damage prevention or attenuation has been performed. The overall goal of this thesis research was to identify specific classes of compounds capable of inducing ENS degeneration. These studies are intended to form a knowledge base upon which further studies can build towards the goal of preventing or attenuating clinical ENS damage.

Clinical ENS damage commonly occurs in association with inflammatory disease states. Recent experimental studies have found a causal relationship between damaging intestinal inflammation and ENS degeneration, however there remains a paucity of information on the specific inflammatory mediators involved in this process. Inflammation is a multi-factorial process that involves large numbers of mediator compounds which may be considered as putative inducers of ENS degeneration. In the context of this thesis work, it was neither feasible nor prudent to investigate the neurodegenerative capacity of such large numbers of inflammatory mediators. A small number of candidate molecules were selected for study based upon their known neurodegenerative actions within other systems, their known involvement in intestinal

inflammatory disease pathogenesis, and initial evidence suggesting roles for these molecules in ENS pathogenesis.

Despite the multitude of compounds involved in inflammation and the complexity of inflammatory processes, specific compounds and processes have been implicated as of primary importance in the pathogenesis of CNS neurons. Reactive oxygen species (ROS), proinflammatory cytokines, and the process of anoxia-reoxygenation have been identified as inducers of CNS neurodegeneration and mediators in the neuropathogenesis of inflammatory diseases. As the ENS bears a considerable functional resemblance to the CNS, these compounds and processes are likely to have similar actions in this nervous system. Many of these mediators have been implicated in the pathogenesis of inflammatory diseases of the bowel, whereas initial studies of some of these mediators suggest their involvement in ENS neuropathogenesis. Thus, the general hypothesis of this thesis is that anoxia-reoxygenation, ROS, and the proinflammatory cytokine tumor necrosis factor-α (TNF) act on the ENS to induce neurodegeneration.

In order to determine the neurodegenerative properties of these compounds and processes, an experimental system was required in which the adult ENS could be studied in isolation, in which both qualitative and quantitative studies could be performed, and which was suitable for toxicological experimentation. As no such system existed at the initiation of this study, the first goal of this research project was to establish such a primary cell culture system from the myenteric plexus of the adult rat ileum, as detailed in **Chapter 2**. In this *in vitro* system, all component cell types were identified, and the enteric neurons were characterized neurochemically, morphologically, and electrophysiologically, demonstrating their high correspondence to their *in vivo*

counterparts. Most importantly, this experimental system was optimized for qualitative and quantitative examination of neurotoxicological insults. A common problem of *in vitro* experiments is substrate degradation upon insult, and for this experimental culture system many culture substrates were screened for their ability to maintain cell adhesion throughout a variety of neurotoxic insults. The cell death mechanisms within identified cell types were characterized as to whether they were apoptotic or necrotic.

In order to maximize the utility of this cell culture system, a process was developed for the cryopreservation storage of dissociated cells. The viability and cellular characteristics of these cryopreserved cells in culture was then assessed and compared to those of freshly plated cultures (as detailed in **Chapter 2**). Cryopreserved ENS cultures were morphologically, neurochemically, and electrophysiologically similar to freshly plated cultures, and no significant differences in cellular survival were observed. Efficient cryopreservation of dissociated neurons is generally considered infeasible, if at all possible. Our process represents the first successful cryopreservation of dissociated enteric neurons for tissue culture. With the development of this highly efficient experimental system, our research into enteric neurodegeneration became significantly more productive.

Initial experiments focused on the identification of specific inducers of enteric neurodegeneration, and characterization of their neurodegenerative properties. The actions of oxidative stress and membrane lipid peroxidation on ENS viability were investigated in **Chapter 2** of this study. 4-hydroxynonenal (HNE), an effector of lipid peroxidation-induced cell damage, acted directly on the ENS to induce cell death. Neurons were more susceptible than glia to such insult, with cell death occurring

predominantly via apoptosis at lower concentrations. Further studies determined the neurotoxicity of specific reactive oxygen species (ROS). Both hydrogen peroxide and the nitric oxide (NO) donor sodium nitroprusside (SNP) displayed dose-dependent toxicities for enteric neurons. Their neurotoxicity was potentiated in combination, presumably due to reaction of these two compounds to form the more toxic product, peroxynitrite. This study represents the first direct evidence for oxidative stress and membrane lipid peroxidation in the induction of enteric neurodegeneration, and implicates apoptosis as a predominant cell death mechanism in such damage.

The process of hypoxia-reoxygenation, which is frequently associated with intestinal inflammatory damage and which involves oxidative stress damage mechanisms, was investigated for its role in enteric neurodegeneration (**Chapter 2**). Although enteric neurons were highly resistant to hypoxia ($O_2 = 2\%$) followed by reoxygenation, neurons were highly sensitive to anoxia ($O_2 \leq 0.01\%$) followed by reoxygenation. Neuronal degeneration in response to anoxia-reoxygenation increased with anoxic exposure. Both anoxia and subsequent reoxygenation induced significant neurodegeneration in enteric cultures. The characteristics of enteric neuron responses to anoxia-reoxygenation insult resemble those of other neural systems, and damage is hypothesized to occur via common intracellular mechanisms. Our observations of *in vitro* anoxia-reoxygenation–induced enteric neurodegeneration suggest that this process may be involved in clinical ENS damage.

Glial cell line-derived neurotrophic factor (GDNF) is a developmental neurotrophin and neuroprotective compound active in both the CNS and peripheral nervous system (PNS). We sought to determine if GDNF, which is also present in the

adult gastrointestinal tract, was capable of protecting enteric neurons against toxic insult (**Chapter 2**). Anoxia-reoxygenation, hydrogen peroxide, SNP, and hydrogen peroxide + SNP, at concentrations that induced approximately 50% neuronal death in ENS cultures, were tested for their neurodegenerative potency in cultures pre-incubated with GDNF. At concentrations neuroprotective in other culture systems, GDNF prevented only that ENS neurodegeneration induced by hydrogen peroxide. Although this represents an initial indication that GDNF might prove to be an *in vivo* ENS neuroprotective agent, our study also demonstrates GDNF's restricted neuroprotective ability. We suggest that, as in other nervous systems, GDNF interferes with a given step in specific apoptotic pathway, thereby preventing apoptosis, but is unable to prevent cell death induced via separate apoptotic pathways, or with downstream events in an apoptotic cascade. Alternatively, GDNF might induce antioxidants that are selectively effective against hydrogen peroxide versus the NO donor SNP.

In **Chapter 3**, the proinflammatory cytokine tumor necrosis factor-alpha (TNF), a molecule of prime importance in inflammatory bowel disease pathogenesis, was investigated to determine its involvement in enteric neurodegeneration. Currently, there is considerable interest in this molecule, whose targeted inactivation has been employed in clinical trails for inflammatory bowel disease treatment. Thus, the functional characteristics of this molecule within the intestine were investigated in addition to its possible role in enteric neurodegeneration.

Recent studies by our collaborators have determined that, in mice pre-exposed to recombinant human TNF (rhTNF), otherwise sub-effective doses of rhTNF induce marked stress responses and illness behaviors. Our research extended these findings to

determine if rhTNF sensitizes intestinal tissues to the effects of further rhTNF exposure (as detailed in **Chapter 3**). We determined that previous exposure to rhTNF sensitizes several intestinal cell types including enteric neurons to the effects of subsequent rhTNF exposure. Through this sensitization effect, otherwise sub-effective doses of rhTNF produce intestinal damage similar to that seen in IBD pathogenesis. Within the intestine, rhTNF demonstrated regional selectivity in its effects, with most responses confined to ileal and colonic regions. This regional selectivity is postulated to reflect intrinsic differences in the susceptibility of different intestinal regions to insult and injury. The sensitization of intestinal tissues and enteric neurons to rhTNF re-exposure was time-dependent. rhTNF is the first cytokine for which a time-dependent sensitization has been found.

In vivo, rhTNF levels that were cytotoxic for intestinal villi epithelia induced functional alterations in sensitized enteric neurons without any accompanying neurodegeneration. As these rhTNF actions occurred in the absence of marked inflammatory cell infiltration, we hypothesized that an inflammatory infiltration of macrophages was required to mediate the degeneration of enteric neurons. To test this hypothesis, the role of macrophages in rhTNF actions on enteric neurons *in vitro* was tested in macrophage-ENS co-culture experiments. Although, in the absence of macrophages, rhTNF did not induce neurodegeneration, similar sensitization treatments with rhTNF in the presence of macrophages induced significant degeneration of enteric neurons.

In summary, the findings of this thesis are consistent with the hypothesis that inflammatory mediators can induce enteric neurodegeneration. This study identified

specific inflammatory mediators as inducers of enteric neurodegeneration, characterized their neurodegenerative properties, and identified GDNF as a neuroprotective agent. Subsequent investigations further defined the respective roles of inflammatory immunocytes and the pro-inflammatory cytokine TNF in enteric neuronal cell death relevant to IBD pathogenesis. These findings are intended to form a basis for further studies into the mechanisms underlying clinical enteric neurodegeneration in disease pathogenesis.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
AIF	apoptosis inducing factor
AMPA	(*S*)-2-Amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid
ANS	autonomic nervous system
AP-1	activator protein 1
ARTN	artemin
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
Cl-	chloride ion
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CO_2	carbon dioxide
DAG	diacylglycerol
DNA	deoxyribonucleic acid
ENK	enkephalin
ENS	enteric nervous system
eNOS	endothelial constitutive NOS
FADD	Fas-associated death domain protein
GABA	gamma aminobutyric acid

GDNF	glial cell line-derived neurotrophic factor
GI	gastrointestinal
GPI	glycosyl-phosphatidyl inositol
HBSS	Hank's balanced salt solution
HCO_3-	bicarbonate
HNE	4-hydroxy-2-nonenal
HOCl	hypochlorous acid
IAP	inhibitor of apoptosis
IBD	inflammatory bowel disease
ICAM-1	intracellular adhesion molecule-1
ICE	interleukin-1β converting enzyme
IFNs	interferons
IL	interleukin
IL-1β	interleukin-1 beta
IL-4	interleukin-4
IL-6	interleukin-6
IL-10	interleukin-10
iNOS	inducible NOS
IP_3	inositol 1,4,5-triphosphate
JNK	c-jun N-terminal kinase
LIF	leukemia inhibitory factor
L-NAME	N^G-nitro-L-arginine methyl ester
MAP	mitogen-activated protein

MAPK	mitogen-activator protein kinase
MLP	membrane lipid peroxidation
NA	noradrenaline
Na^+/K^+-ATPase	sodium potassium ATPase
NAD/NADPH	nicotinamide adenine dinucleotide
NFκB	nuclear factor kappa B
NGF	nerve growth factor
NIK	nuclear factor κB-inducing kinase
NMDA	*N*-methyl-d-aspartate
nNOS	neuron constitutive NOS
NO	nitric oxide
$NO_2^{\cdot}$	nitrogen dioxide
NOS	nitric oxide synthase
NPY	neuropeptide Y
NT	neurotrophin
NTN	neurturin
O_2	molecular oxygen
$O_2^{\cdot}$	peroxide radical
$OH^{\cdot}$	hydroxyl radical
$ONOO^{\cdot}$-	peroxynitrite
PACAP	pituitary adenylyl cyclase activating peptide
PAF	platelet activating factor
PARP	poly (ADP-ribose) polymerase

PSP	persephin
PTP	permeability transition pore
RAIDD	RIP-associated ICH-1/Ced-3 homologous death domain protein
RIP	receptor-interacting protein
RO·	alkyl radical
ROO·	peroxyl radical
ROS	reactive oxygen species
TGF-β	transforming growth factor-beta
TNBS	trinitrobenzene-sulfonic acid
TNF	tumor necrosis factor
TNF-BP	TNF-binding protein
TNFR	TNF receptor
TRADD	TNF-receptor 1 associated death domain containing protein
TRAF 2	TNF-receptor 1 associated protein 2
TRAIL-R1	TNF-related apoptosis-inducing ligand–receptor 1
TRAIL-R2	TNF-related apoptosis-inducing ligand –receptor 2
TRAMP	TNF-receptor-related apoptosis-mediating protein
Trk	receptor tyrosine kinase
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
VCAM-1	vascular cell adhesion molecule-1
VIP	Vasoactive intestinal peptide

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Chapter 1. INTRODUCTION

The gastrointestinal tract serves to digest and absorb ingested nutrients and liquids, to protect the body against foreign contaminants and infectious agents, and to eliminate waste from the body. Control of this complex organ system is dependant upon an intrinsic enteric nervous system (ENS) which coordinates all gut behavior including gut motility, mucosal transport, microcirculation, and local immune functions. The CNS can modulate these behaviors by interaction with the intrinsic enteric innervation. However, disruption of the brain-gut axis does not in the long term prevent normal gut function. In contrast, damage to the ENS produces long-term impairment or permanent alterations in gastrointestinal function. Typical clinical symptoms of enteric neural damage include disordered or impaired gastrointestinal movement, nausea, vomiting, weight loss and malnutrition. These symptoms can occur in gut diseases such as IBD, and as secondary complications in a variety of other diseases including Parkinson's, myotonic dystrophy, and diabetes. Despite the potentially broad application of neuroprotective therapies for the treatment of gastrointestinal dysfunction, treatment to prevent or attenuate ENS damage is not currently available. Identification of the neurodegenerative moieties underlying ENS neuropathology and their damage mechanisms would enable the development of such neuroprotective therapies. This represents the underlying rationale for my doctoral research project.

An intriguing feature of clinical enteric neurodegeneration is its association with gut inflammation (Quigley, 1996; Edwards et al., 1999; Kubota et al., 1992; Schuffler, 1984; Schuffler et al., 1981). Given that inflammatory mediators induce neurodegeneration in the CNS, which is largely protected from somatic peripheral

influences by the blood-brain barrier, it follows that the ENS, with its comparatively weak protective barrier and its position within the inflammation-prone intestinal wall, would be at much greater risk for such damage. The general hypothesis of this thesis was thus that specific inflammatory mediators induce enteric neurodegeneration. Indeed, within recent studies using animal models of IBD, intestinal inflammation has been causally linked to enteric neurodegeneration (Palmen et al., 1998; Sanovic et al., 1999), although specific mediators directly involved in such processes have not been identified.

In this thesis, an experimental system was developed to facilitate study of neurotoxicity in the ENS. This culture system was used for determination and characterization of the neurotoxic properties of specific inflammatory components identified as putative mediators of enteric neurodegeneration. As background to these studies, the structure and function of the ENS must be known, as must the characteristics of its damage. In this introduction, specific inflammatory mediators are also presented whose roles in such ENS damage are examined in this thesis. This introduction begins with a brief overview of the roles of the enteric nervous system in gastrointestinal function and the consequences of its degeneration, which is the impetus for the experimental investigation of this process.

1.1 Innervation of the Gastrointestinal Tract

The gastrointestinal (GI) tract contains three physiological effector systems: visceral smooth muscle, which is responsible for motility; the intestinal mucosa, which is responsible for fluid and electrolyte homeostasis; and the vasculature (Surprenant, 1994). The neural control of these effector systems is supplied by both extrinsic autonomic nerves and by neurons intrinsic to the gut wall. The intrinsic innervation of the GI tract is

extensive and includes sensory neurons, interneurons, and motor neurons that are integrated into an independent nervous system known as the ENS. The extrinsic innervation consists of sympathetic and parasympathetic autonomic nerves that interconnect the central nervous system (CNS) with the ENS, forming the brain-gut axis (Camilleri, 1990).

The brain-gut axis provides for both cognitive and autonomic influence over GI activity. The ENS coordinates GI activity in widely separate regions of the GI tract, while the extrinsic innervation adjusts GI activity according to local factors and to the physical and chemical nature of ingested foodstuffs (Taylor and Bywater, 1988). This extrinsic innervation is mainly involved in the rapid induction of digestive enzymes within the mouth and stomach in response to the presence of food, and with the conscious control of defecation. Extrinsic innervation exerts more modulatory control over GI function in certain gut regions than in others. For example, parasympathetic vagal stimulation strongly influences digestion and motility in the stomach, but plays a modest role in regulating these functions in the small intestine. Additionally, although sympathetic and parasympathetic innervation is essential for regulation of those portions of the esophagus and the external anal sphincter which are invested with striated muscle, extrinsic innervation is relatively unimportant in the control of GI tract smooth muscle function (Camilleri, 1990).

Autonomic efferents to the gastrointestinal tract terminate predominantly upon intrinsic enteric neurons. Thus, the actions of the autonomic nervous system (ANS) on GI motility are indirect, and occur primarily through ANS modulation of ENS function. For example, parasympathetic efferents converge together with local enteric axons onto

myenteric cholinergic motor neurons, which in turn stimulate contraction of gut smooth muscle. The sympathetic nervous system acts together with other enteric neural circuits to inhibit motor activity (Minaire, 1989). Impairment of ENS function can directly affect normal gut function and also disrupt CNS modulation of GI activity. Although extrinsic innervation plays an important role in regulating the gastrointestinal tract, the intrinsic ENS innervation plays the predominant role in neuronal modulation of GI function. Even in the complete absence of extrinsic innervation, complex reflex activities involving GI motility, intestinal ion transport, and mucosal blood flow occur. However, when damage or degeneration occurs within the ENS, both those GI functions directly controlled by the ENS, and those modulated by the CNS are compromised.

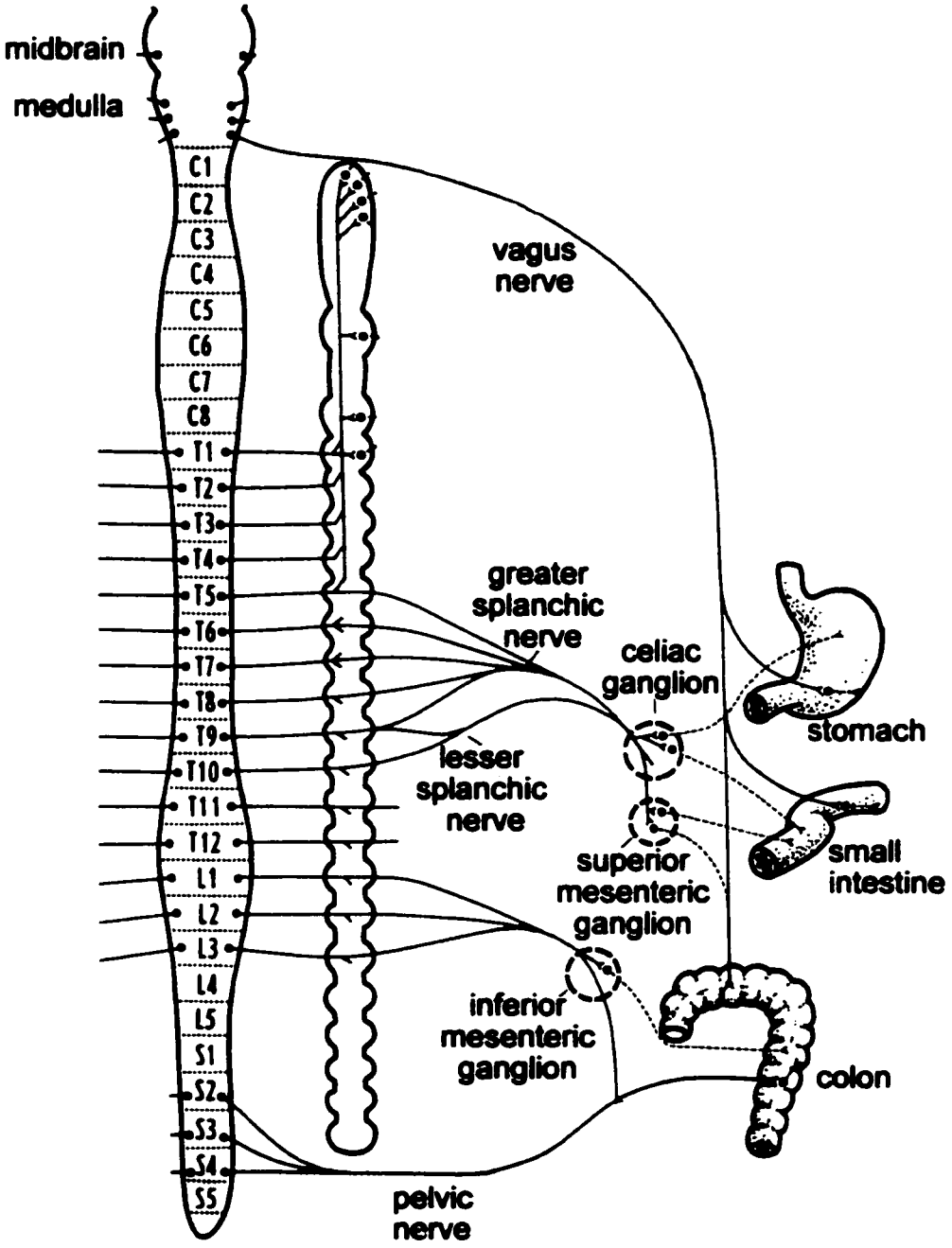
1.1.1 Autonomic Innervation of the GI tract

The autonomic nervous system is predominantly an efferent system transmitting impulses from the CNS to peripheral organ systems, including the GI tract. Autonomic nerves constitute nearly all of the efferent fibres that leave the CNS. However, afferent fibres are also present within both the parasympathetic and sympathetic nerve bundles. These fibres project back to the CNS to provide sensory input for integration (Johnson, 1991). The two divisions of the ANS are antagonistic in their actions. Sympathetic innervation is involved in "fight-or-flight" responses, in which the sympathetic nervous system up-regulates sweating, increases heart beat, and inhibits digestion. In contrast to the "fight-or-flight" activities of the sympathetic nervous system, the parasympathetic nervous system enhances "rest-and-digest" activities that conserve and restore body energy. These activities include digestion and absorption of foodstuffs. Normally, these

two divisions are in balance, with the activity of one or the other becoming dominant as dictated by the needs of the organism.

Parasympathetic innervation drives peristaltic contractions within the GI tract via release of the excitatory neurotransmitter acetylcholine (ACh) onto enteric motor neurons, which in turn releases ACh onto smooth muscle (Furness and Costa, 1987). Preganglionic parasympathetic neurons of the medulla project axons that travel through the vagus nerve (cranial nerve X) to synapse upon intrinsic neurons of the myenteric plexus in the stomach, small intestine, and anterior colon. Preganglionic parasympathetic neurons of the sacral spinal cord project axons that synapse onto intrinsic neurons within myenteric plexus ganglia of the posterior colon and rectum (McConalogue and Furness, 1994) (Figure 1).

Sympathetic tone inhibits gastrointestinal motility and digestion. Neurons of the thoracic and lumbar spinal cord regions give rise to the preganglionic component of the sympathetic innervation to the GI tract. Their axons traverse the sympathetic chain ganglia to synapse with second order neurons within prevertebral ganglia (celiac and mesenteric ganglia). Axons of the greater and lesser splanchic nerves synapse with postganglionic neurons of both celiac and superior mesenteric ganglia, while those axons emanating from lumbar spinal cord regions synapse with postganglionic neurons within the inferior mesenteric ganglion. Neurons from the celiac ganglion innervate the stomach and small intestine, those from the superior mesenteric ganglion innervate the small intestine and anterior colon, and those from the inferior mesenteric ganglion innervate the posterior colon (Figure 1). The postganglionic axonal projections from these neurons terminate directly on their intestinal targets including glandular tissue, smooth muscle


midbrain
medulla
C1
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S1
S2
S3
S4
S5
vagus nerve
greater splanchic nerve
celiac ganglion
stomach
lesser splanchic nerve
superior mesenteric ganglion
small intestine
inferior mesenteric ganglion
colon
pelvic nerve

Figure 1. Extrinsic innervation of the gut. Sympathetic neurons are shown in red; while parasympathetic components are in blue. Solid lines represent preganglionic fibers; broken lines, postganglionic fibers. Figure adapted from Carpenter, 1976.

cells, and enteric neurons (McConalogue and Furness, 1994). The inhibitory neurotransmitter noradrenaline (NA), released from postganglionic sympathetic neurons, can inhibit GI smooth muscle contraction, acid secretion in the stomach, blood flow in the small and large intestines, and intestinal secretion of both water and electrolytes (Furness and Costa, 1987).

1.1.2 Intrinsic Enteric Innervation

The intrinsic enteric nerves were originally considered a division of the autonomic nervous system (ANS). It is now recognized that the enteric nervous system operates as an independent integrative nervous system with structural and functional properties similar to the CNS (Wood, 1994). Neurons in the enteric nervous system (ENS) are in a unique environment: they are regularly distorted by peristaltic contractions (Gabella, 1990) and exposed to mild inflammatory insults (Collins, 1996), and there is a constant remodeling of the neural connections in the mucosa due to epithelial sloughing. This environment provides cell phenotypes that differ substantially from other autonomic neurons (Giaroni et al., 1999). Unlike the sympathetic and parasympathetic nervous systems, which are highly innervated by CNS neurons, only 1-2,000 nerve fibres connect the brain to the approximately 1×10^8 enteric nerve cells in small intestine, and thus the vast majority of enteric nerve cells receive no direct connections from the brain or spinal cord (Kirchgessner and Gershon, 1989). CNS control of the ENS is thus limited, acting primarily to modify preprogrammed enteric circuits at the level of the enteric plexuses (Wood, 1991), and indeed the ENS can function independently of CNS input (Gershon, 1990). The ENS ultimately controls all gut behavior including the motility (Costa and Brookes, 1994; Furness and Bornstein, 1995), exocrine and endocrine secretions (Cooke,

1994), and microcirculation (Surprenant, 1994) of the gastrointestinal tract, and is involved in regulating immune and inflammatory processes (Lundgren et al., 1989). ENS neural circuits coordinate and integrate these multiple cellular activities of motor, secretory, vascular, and absorptive cells within distinct layers of the gut wall (Costa and Brookes, 1994).

Thus, both extrinsic autonomic nerves and intrinsic enteric nerves innervate the GI tract to control its function. Although extrinsic and intrinsic control of the GI tract can be coordinated, the ENS can function independently of extrinsic innervation and is essential for the ongoing control of all gut behaviors.

1.2 The Enteric Nervous System

1.2.1 Enteric Neural Plexuses

Enteric neurons are organized into two main ganglionated neural plexuses whose localization within the GI tract are shown diagrammatically in Figure 2. The myenteric plexus is located between the circular and longitudinal muscle layers, while the submucosal plexus is found between the circular muscle layer and the muscularis mucosae. This latter plexus contains from one to three smaller ganglionated plexuses depending upon the species, generally referred to as Henle's, Meissner's, and the intermediate plexuses (Furness and Costa, 1987). The myenteric plexus, considered to be the primary nerve network of the gut wall, is composed of significantly more neurons, larger ganglia, and more complex interganglionic connections than are found in the submucosa (Gershon et al., 1994). The smaller submucosal nerve networks are most well developed in the small intestine (Goyal and Hirano, 1996). Although the myenteric and submucosal plexuses are specialized for the control of gastrointestinal motility and

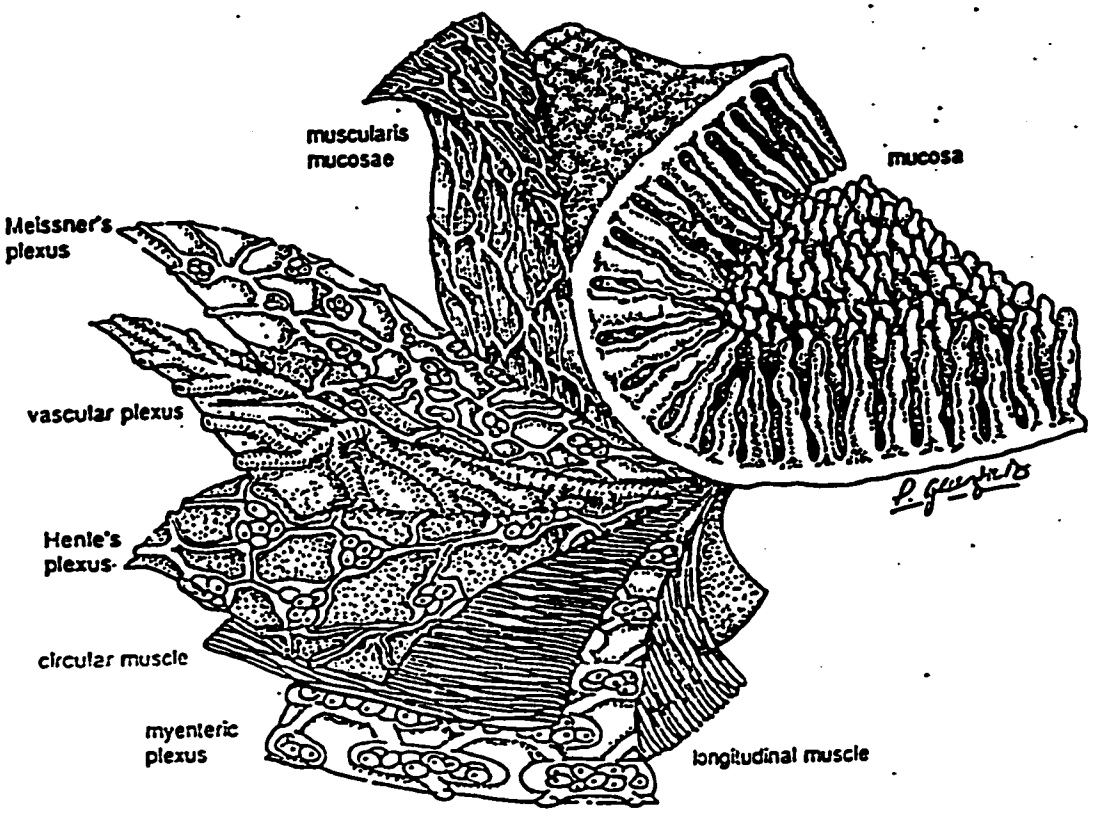

muscularis
mucosae
mucosa
Meissner's
plexus
vascular plexus
Henle's
plexus
circular muscle
myenteric
plexus
longitudinal muscle

Figure 2. Diagrammatic representation of the layers of the intestinal wall. Note the myenteric plexus between the circular and longitudinal muscle layers. The submucosal plexus, located between the muscularis mucosae and the circular muscle, is shown as its two components – Henle's and Meissner's plexuses. Figure by A. Krantis.

intestinal ion transport respectively, these plexuses are interconnected and are ultimately functionally integrated through the myenteric plexus (Furness and Costa, 1987).

1.2.2 Enteric Ganglia

Enteric ganglia are composed exclusively of enteric neurons, glial cells, and their processes, all covered by a thin layer of basal lamina. A unique feature of enteric ganglia is that they do not contain blood vessels, connective tissue, or collagen fibrils (Gershon et al., 1994). Enteric glial cells resemble CNS astrocytes (Jessen and Mirsky, 1980) and as such these cells do not wrap around individual axons, rather, entire bundles of axons are fitted into the invaginations of enteric glia (Gershon, 1999). That the targeted ablation of enteric glia leads to degenerative changes in the ENS, epithelial degeneration and sloughing, inflammatory infiltration of the mucosa, and intraluminal hemorrhage (Bush et al., 1998; Sofroniew et al., 1999), suggests that enteric glia possess some factor that is immunosuppressive and anti-inflammatory under physiological conditions.

1.2.3 Enteric Neurons

Enteric neurons are commonly classified according to their functional characteristics, axonal projections, morphology, electrophysiological properties, and neurochemical characteristics (Costa et al., 1998). In both the small and large intestine, each neuronal subtype has its own characteristic distribution and projection pattern (Ekblad et al., 1988). As in the CNS, more than one transmitter or neuron-specific marker may be expressed by a single enteric neuron, with many putative neurotransmitters colocalizing in a variety of combinations (Ekblad et al., 1984; Sternini, 1988).

1.2.4 Classification of Enteric Neurons

Enteric neurons are classified functionally as intrinsic sensory neurons, interneurons, motor and secretomotor neurons. Enteric ganglia are "mixed ganglia" and contain all of these types of neurons in combination and form interconnecting pathways between individual ganglia (see Figure 3). Intrinsic sensory neurons predominate within the submucosal nerve networks, although they are also present within the myenteric plexus. Sensory neurons in the myenteric plexus are stimulated in response to mechanoreceptor activation within the muscle layers. In contrast, submucosal sensory neurons represent the full spectrum of sensation detection, with their receptors being stimulated by mechano- and chemoreceptors in the mucosa and mechanoreceptors in muscle layers (Minaire, 1989) (see Figure 3). It is not currently known whether submucosal sensory neurons themselves act as mechanoreceptors, or if these neurons are stimulated secondary to the activation of mechanosensitive enterochromaffin cells in the mucosal epithelium (Gershon, 1999).

Interneurons relay signals from sensory neurons to motor or secretomotor neurons. Interneurons also form multiple excitatory connections with each other to create ascending and descending chains that run along the intestine (Kunze and Furness, 1999). Within the myenteric plexus, interneurons receive signals from sympathetic and parasympathetic neurons, and from enteric sensory neurons from the same or different ganglia, within a given plexus, or from different enteric plexuses (see Figure 3). While motor neurons of the myenteric plexus act on both longitudinal and circular muscle layers to control intestinal movements, and, to a small extent, on vascular smooth muscle cells that control blood flow, secretomotor neurons of this plexus either act indirectly through

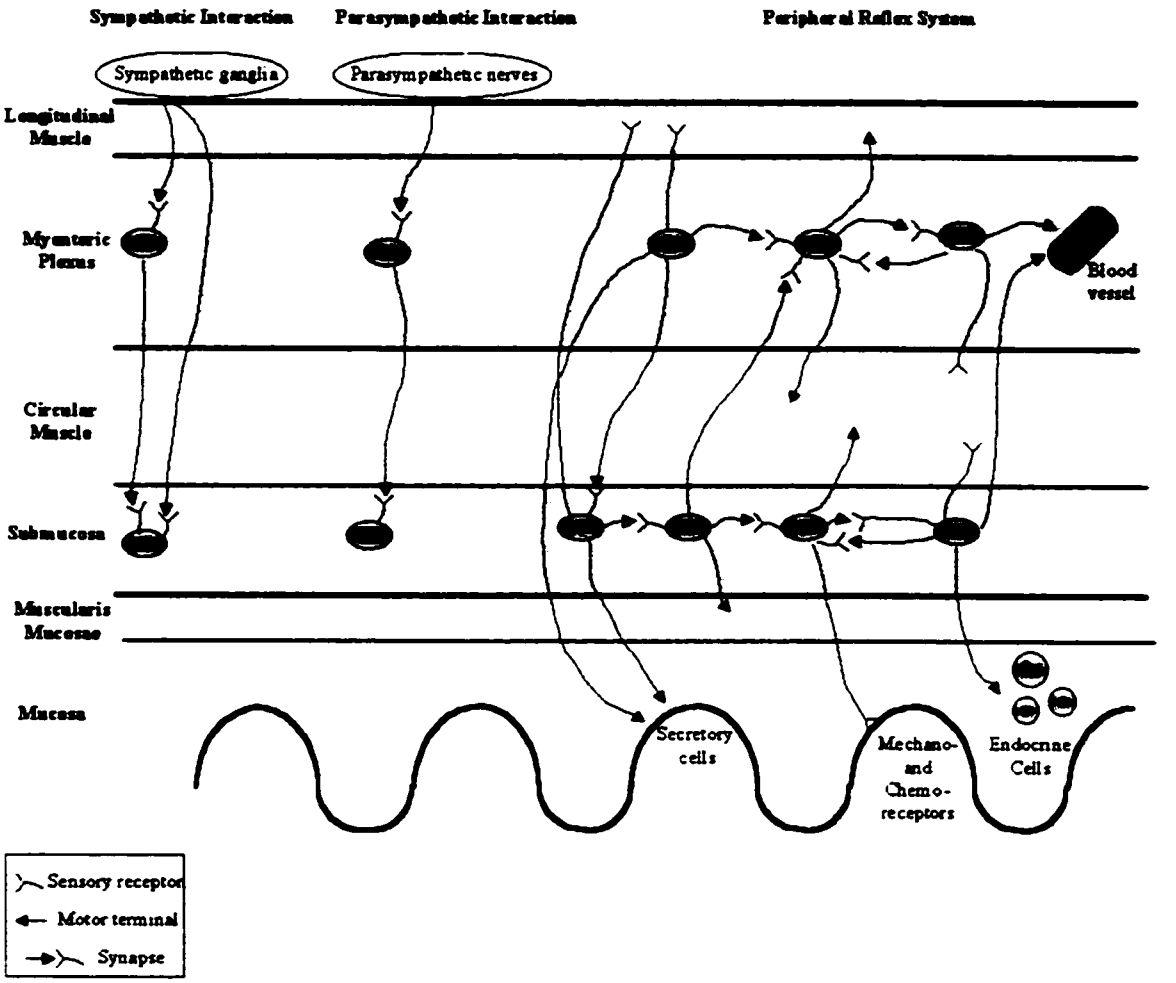

Sympathetic Interaction
Parasympathetic Interaction
Peripheral Reflex System
Sympathetic ganglia
Parasympathetic nerves
Longitudinal Muscle
Myenteric Plexus
Blood vessel
Circular Muscle
Submucosa
Muscularis Mucosae
Mucosa
Secretory cells
Mechano- and Chemo-receptors
Endocrine Cells
Sensory receptor
Motor terminal
Synapse

Figure 3. Simplified representation of ENS neural circuits. This diagram shows the sensory neurons, interneurons, and motor/secretomotor neurons, and their connections both within the enteric nervous system and with the autonomic nervous system. Both myenteric and submucosal neurons can receive direct input from sympathetic ganglia. These enteric neurons can in turn interact with each other, and with surrounding tissue. Only neurons of the myenteric plexus can receive direct innervation from parasympathetic nerves, however. Neurons of both the myenteric and submucosal plexuses can innervate a number of local target cells and tissues. Neurons in both these plexuses can innervate longitudinal and circular muscle, villi, secretory cells, blood vessels, and other neurons within both plexuses. Only submucosal neurons, however, can innervate mucosal endocrine cells and the muscularis mucosae. While sensory neurons of both plexuses can receive input from the longitudinal and circular muscle layers and from other enteric neurons within both plexuses, only submucosal neurons can receive input from mechano-and chemo-receptors within villi endothelium.

submucosal neurons or directly on mucosal epithelium to control secretion and absorption (Furness et al., 1989; Kirchgessner and Gershon, 1990; Szuszewski and Miller, 1994) (see Figure 3). Submucosal neurons can also act on longitudinal and circular muscle layers, and the muscularis mucosae to influence intestinal movements (Minaire, 1989). However, the primary function of secretomotor and motorneurons of the submucosa is immune regulation and the control of mucosal function. These neurons innervate secretory cells of the mucosal epithelium, immunomodulatory and inflammatory cells of the gut that are involved in mucosal, immunologic, allergic, and inflammatory responses, and vascular smooth muscle cells that control blood flow (Goyal and Hirano, 1996) (see Figure 3).

Enteric neurons were first classified according to their morphological differences (Dogiel, 1899). Based on their differing shapes of their neuronal somata, as well as the number and type of axons or dendrites emanating from them, Dogiel identified three morphological neuron types within the ENS. Of these, Dogiel types I and II neurons comprise the vast majority of enteric neurons (Browning and Lees, 1996). The three characteristic neuron shapes originally identified by Dogiel have more recently been revised to include eight morphological classes of enteric neurons (Stach, 1989). Stach expanded the number of identified neuron types to eight by expanding the identifying criteria examined to include the direction of neurite projections within the bowel, cell relationships to each other, cell topography, the regional distribution of the neurons, the identification of vascularization and synaptic inputs, neurochemical correlations, and the electrophysiological characteristics of each neuron type. This work initiated correlation of neuronal morphology within the ENS (Gershon et al., 1994).

Enteric neurons are also classified into subtypes dependent upon the neurotransmitters that they express. It is now generally recognized that at least 30 different classes of chemicals transmit instructions in the brain, and that all of these classes are also represented in the ENS (Gershon, 1998; Costa et al., 1998). However, although many substances have been suggested to be enteric neurotransmitters based on pharmacological groups or due to evidence of their presence in the ENS, to date very few substances have been experimentally established as ENS neurotransmitters. As enteric neurons commonly co-localize several transmitters and candidate transmitters, neurons can be functionally classified based on the distinct combinations of substances that they co-localize. For example, in the mouse small intestine, NOS/VIP $\pm$ NPY compromise approximately 25% of all myenteric neurons (Sang and Young, 1996). By these means, over 30 neurochemical subtypes have been identified within the ENS (McConalogue and Furness, 1994). The distribution of these neurochemical subtypes of neurons varies both between species and between different GI regions (Schemann et al., 1995; Furness et al., 1990; Ekblad et al., 1991; Wattchow et al., 1998; Sang and Young, 1996). Although such variations have been characterized, their functional significance is poorly understood (McConalogue and Furness, 1994).

Although acetylcholine (ACh) is commonly recognized as the primary excitatory transmitter in enteric neurons, glutamate and tachykinins are also suggested to act as excitatory neurotransmitters in this nervous system (Liu et al., 1997; McConalogue and Furness, 1994). Vasoactive intestinal peptide (VIP), adenosine triphosphate (ATP), and nitric oxide (NO) are common ENS inhibitory transmitters. Additional putative inhibitory neurotransmitters include pituitary adenylyl cyclase activating peptide

(PACAP), neuropeptide Y (NPY), and enkephalin (ENK) (McConalogue and Furness, 1994). In the ENS, gamma aminobutyric acid (GABA) seems to act as both an excitatory and an inhibitory neurotransmitter (Krantis et al., 1980; Krantis et al., 1998), depending on the type of GABA receptor that is activated postsynaptically.

The electrophysiological properties of enteric neurons have also been used to define subtypes of enteric neurons. Two neuronal subtypes have thus been identified; the S/type 1 (so-called 'spiking neurons') and the AH/type 2 neurons (so-called 'after-hyperpolarizing neurons') (Holman et al., 1972; Hirst et al., 1974). S/Type 1 neurons, when stimulated, possess a characteristically spiked action potential, in contrast to the pronounced shoulder on the falling phase of the action potential characteristic of Type AH/2 neurons. Type AH/2 neurons also undergo prolonged hyperpolarizing afterpotentials following action potential generation, which prevent them from repetitively discharging actions potentials as is characteristic of Type S/1 neurons. These two electrophysiological neuronal subtypes are commonly accepted to correspond to both morphological and functional neuronal subtypes. Type S/1 neurons are defined as motor and interneurons with Type I Dogiel morphology, whereas Type AH/2 neurons are sensory neurons with Type II Dogiel morphology (Bornstein et al., 1994; Browning and Lees, 1996). There are many functional and neurochemical subtypes of Type I neurons, each with their own characteristic chemical coding. Excitatory motor neurons commonly express Substance P and ACh (as indicated by immunoreactivity for its synthesizing enzyme choline acetyltransferase), while VIP and NO are localized to inhibitory motor neurons. Descending interneurons commonly express ChAT immunoreactivity in combination with several possible neurochemicals, and ascending interneurons express

ChAT, calretinin and tachykinin immunoreactivity. Type II neurons characteristically express some combination of ChAT, tachykinin, and calbindin immunoreactivity (Furness, 2000; Costa et al., 1996). Correlations between ENS morphology, function and chemical coding are detailed in Table 1. Data in this table represents that of the guinea pig small intestine, in which region all classes of neuron have been identified and characterized (Furness et al., 1994; 2000; Costa et al., 1996).

One of the major aims of gastrointestinal neurobiology has been to relate the properties and connections of enteric neurons to the functions that they control. To achieve this aim, a complete neuroanatomical identification of the different classes of enteric neurons is essential (Costa et al., 1996). In the small intestine of the guinea pig, all of the types of enteric neurons have been identified in terms of their morphologies, projections, primary neurotransmitters and physiological properties (Furness et al., 1994; 2000; Costa et al., 1996). In this region there are 14 functionally defined neuron types, each of which has characteristic morphological, neurochemical and biophysical properties (Costa et al., 1996). By such means, the ENS has been demonstrated to possess a unique and characteristic structure, whose component cells can be identified by their functional, chemical, and morphological properties. Identification of these defining characteristics is intrinsic to the evaluation of the physiological relevance of *in vitro* models, as performed in **Chapter 2** of this thesis.

1.3 Enteric Neurodegeneration

1.3.1 ENS Susceptibility To Damage

Within the CNS, both endogenous and exogenous proteins are incapable of permeating the parenchyma of the brain, except in circumventricular areas, due to the

Percentage	Chemical Coding	Morphology	Functional Class	Comments
Myenteric plexus (note: percentages represent that of all myenteric neurons. Myenteric neurons represent greater than 97% of all enteric neurons present within the guinea pig ileum).				
12%	Short: ChAT/TK/ENK/GABA Long: ChAT/TK/ENK/NFP	Type I	Excitatory circular muscle motor neurons	To all regions, primary transmitter ACh, cotransmitter TK
16%	Short: NOS/VIP/PACAP/ENK/NPY/GABA Long: NOS/VIP/PACAP/dynorphin/BN/NFP	Type I	Inhibitory circular muscle motor neurons	Several cotransmitters with varying prominence
25%	ChAT/calretinin/TK	Type I	Excitatory longitudinal muscle motor neurons	Primary transmitter ACh, cotransmitter TK
2%	NOS/VIP/GABA	Type I	Inhibitory longitudinal muscle motor neuron	Several cotransmitters with varying prominence: NO, ATP, VIP, PACAP
5%	ChAT/calretinin/TK	Type I	Ascending interneurons (local reflex)	Primary transmitter ACh
5%	ChAT/NOS/VIP±BN±NPY	Type I	Descending interneurons (local reflex)	Primary transmitter ACh, ATP may be a cotransmitter
2%	ChAT/5-HT	Type I	Descending interneurons (secretomotor reflex)	Primary transmitters ACh, 5-HT
4%	ChAT/SOM	Type I	Descending interneurons (migrating myoelectric complex)	Primary transmitter ACh
26%	ChAT/calbindin/TK/NK_1 receptor	Type II	Myenteric intrinsic primary afferent neurons	Primary transmitter TK
<1%	ChAT/BN/VIP/CCK/ENK	?	Intestinofugal neurons (to prevertebral ganglia)	Primary transmitter ACh
Submucosal plexus (note: percentages represent that of all submucosal neurons. Submucosal neurons represent less than 3% of all enteric neurons present within the guinea pig ileum).				
45%	VIP/GAL	Type III	Non-cholinergic secretomotor / vasodilator neurons	Primary transmitter VIP. A small proportion of these have cell bodies in myenteric ganglia
15%	ChAT/calretinin/dynorphin	Type III	Cholinergic secretomotor / vasodilator neurons	Primary transmitter ACh
29%	ChAT/NPY/CCK/SOM/CGRP/dynor-phin	Type III	Cholinergic secretomotor (non-vasodilator neurons)	Primary transmitter ACh. A small proportion of these have cell bodies in myenteric ganglia
11%	ChAT/TK/calbindin	Type II	Submucosal intrinsic primary afferent neurons	Primary transmitter assumed to be TK

Table 1. Types of neurons in the enteric nervous system. This table lists the neuron types that are found in the guinea-pig small intestine, some of their defining characteristics, and percentages of occurrence in each of the ganglionated plexuses. (as adapted from Furness, 2000 and Costa et al., 1996). Abbreviations: BN, bombesin a.k.a. GRP: CCK, choleocystokinin; ChAT, choline acetyltransferase; CGRP, calcitonin gene related peptide; ENK, enkephalin; GABA, gamma amino butyric acid; GAL, galanin; GRP, gastrin releasing peptide; 5-HT, 5-hydroxytryptamine; NFP, neurofilament protein; NK, neurokinin; NOS, nitric oxide synthase; NPY, neuropeptide Y; PACAP, pituitary adenyl cyclase activating peptide; SOM, somatostatin; TK, tachykinin; VIP, vasoactive intestinal peptide.

protective blood-brain barrier. In this system, the endothelial cells of brain capillaries form a continuous band of tight junctions that prevent the free diffusion of most substances between the cerebrospinal fluid and blood (Brightman and Reese, 1969). Within sympathetic and parasympathetic nerves, a combination of tight junctions between endothelial cells of penetrating capillaries and tight junctions between perineurial fibroblasts surrounding the nerve also form a protective barrier (Haines, 1997). Within the enteric ganglia, glia form a protective periganglionic sheath over enteric neurons. However, the protective periganglionic sheath formed by enteric glia is neither equivalent to the perineurium barrier present in the ANS, nor to the highly impermeable blood-brain barrier of the CNS. The enteric periganglionic sheath does not fully cover enteric neurons; large areas of neuronal cell bodies and dendrites lie directly beneath the basal lamina and the connective tissue surrounding ganglia (Gabella, 1982). Although the enteric ganglia are avascular, many vessels are concentrated outside their surrounding basal lamina (Gabella, 1972). These vessels are far less permeable than those of the other layers of the bowel wall and so limit the access of exuded molecules to the ENS (Gershon et al., 1994). The enteric basal lamina is also thought to be compact enough to prevent the access of large colloidal particles to enteric neurons (Allen and Kiernan, 1994). However, plasma proteins, that do not generally penetrate the highly impermeable blood-brain barrier, pass from the blood into all parts of enteric ganglia with no evidence that tight junctions between glial or smooth muscle cells prevent these proteins from coming into contact with enteric neurons (Allen and Kiernan, 1990; 1994; Goldstein and Betz, 1986). During inflammation, the extensive exudation of fluid from permeabilized blood vessels that permeates throughout the gut wall can access enteric

ganglion cells. By these means, enteric neurons and glial cells can be exposed to damage by exogenous toxic substances (Kiernan, 1996).

1.3.2 Clinical Symptoms of Enteric Neuropathy

The smooth muscle of the gastrointestinal tract has an intrinsic rhythmicity and does not require neural input to contract. However, as detailed previously, both extrinsic and intrinsic innervation modulate GI tract function. The ENS itself acts upon enteric smooth muscle to organize and help control the rate of gastrointestinal peristalsis, and mediates much of the CNS modulation of GI motility. Destruction of the myenteric plexus impairs these neural functions, and the remaining spontaneous smooth muscle contractions are insufficient for propulsion of bowel contents through the intestinal tract. ENS damage can also inhibit and impair intestinal blood flow, local immune responses, and control of gastrointestinal secretion and absorption when neural modulation of these processes is affected (Giaroni et al., 1999). Thus, clinical disorders associated with the degeneration of enteric neurons are characterized by disturbances in gastrointestinal transit, functional obstruction, tissue damage, and/or nutrient absorption. Symptoms may include impaired esophageal transport, delayed gastric emptying, abdominal distention, reduced peristalsis, disordered small bowel movement, atony of the large bowel, nausea, vomiting, and, in advanced stages of disease, weight loss and malnutrition (Oehmichen and Reifferscheid, 1977; Mathias and Clench, 1995; Clarke et al., 1979).

1.3.3 Morphological Characteristics of Enteric Neuropathy

A number of characteristic morphological changes occur in the damaged ENS. Nerve fibres may display hyperplasia, decreases in fibre density, or fibre damage. In general, damaged ENS nerve fibres appear swollen and frayed, with their parallel courses

transformed into net-like, unorganized patterns (Oehmichen and Reifferscheid, 1977; Kubota et al., 1992). Enteric ganglia may be completely lost or much reduced in cell number, reflecting neuronal cell death (Oehmichen and Reifferscheid, 1977; Sanovic et al., 1999). Within remaining ganglia, neurons of normal appearance are frequently located adjacent to those that are visibly damaged, suggesting intrinsic differences in neuronal subtype susceptibility to damage.

1.3.4 Enteric Neurodegeneration In Disease

Damage and/or degeneration of enteric neurons occurs within a number of intestinal diseases and pathologies (see Table 2). Clinically, ENS damage is frequently found in correlation with intestinal inflammation or inflammation-associated conditions. For example, damage to the adult ENS has been commonly noted within inflammatory bowel disease, a chronic condition characterized by recurring uncontrolled intestinal inflammation (Geboes and Collins, 1998); within idiopathic chronic constipation, a colonic motility disorder thought to result from autoimmune attack targeted to neuronal elements of the ENS (Wood, 2000); and within chronic intestinal pseudo-obstruction, in which intestinal obstruction occurs in the absence of mechanical blockage (Schuffler, 1981). While chronic intestinal pseudo-obstruction can result from abnormalities of either the intestinal smooth muscle or the myenteric plexus (Schuffler, 1984; Schuffler et al., 1981), cases involving myenteric plexus disruption are characterized by inflammatory infiltration of this plexus (Chinn and Schuffler, 1988).

Damage and/or degeneration of the ENS can also occur as a secondary injury in a wide variety of extra-intestinal diseases (see Table 2). Within myotonic dystrophy, an

DISEASE	Myenteric Nerve Cell Damage	Submucosal Nerve Cell Damage	Neuronal types preferentially damaged	Neuronal subtypes selectively spared	Fibre hyperplasia	Fibre damage	Pathogenesis of enteric neuropathy
Chronic intestinal pseudo-obstruction	Yes (1)	No (1)	?	?	No (1)	Yes (1)	Intestinal Inflammation (?) (1b)
Idiopathic chronic constipation	Yes (2)	Yes (2)	VIP (?) (2)	NOS (?) (2)	No (2)	Yes VIP (2)	?
Inflammatory bowel disease	Yes (3)	Yes (3)	?	?	?	Yes (3, 3b) VIP	Intestinal Inflammation (?) (3)
Myotonic dystrophy	Yes (4)	No (4)	Substance P Enk (4)	VIP, NPY (4)	?	Yes(4)	?
Parkinson's disease	Yes (5)	Yes (5)	Dopaminergic (5b) VIP (5c)	?	?	?	Oxidative & free radical damage (?) (5d)
Diabetes mellitis *	Yes (3)	Yes (3)	SOM in submucosal plexus (3)	ACh in myenteric plexus (3)	Yes - galanin in myenteric plexus (3)	Yes (3)	Ischemia (?) Neurotrophic factor deprivation (?)
Chagas' disease	Yes (7)	Yes (7)	NOS activity (7b)	?	?	?	Bacterial infection (?)

Table 2. Disease-specific characteristics of enteric neuropathology. Damage to the enteric nervous system varies with respect to the specific neural plexuses affected, which neural subtypes are involved, whether fibre hyperplasia or degeneration occur, and the underlying damage mechanisms. Within this table "?" denotes that the information is not available, while "(?)" denotes that the information is putative. * denotes that for diabetes mellitis, all characterization of ENS damage has been performed only in animal models.

(1) Chin and Schuffler, 1988
(1b) Wood, 2000
(2) Cortesini et al., 1995
(3) Dvorak et al., 1980; Steinhoff et al., 1988; Lindgren et al., 1991
(3b) Kubota et al., 1992 Note: it was not specifically determined whether the marked decrease in VIP-immunoreactive fibres in IBD patients was due to decreased expression of VIP within nerve fibres or to degeneration of VIP-containing nerve fibres.
(4) Yoshida et al., 1988
(5) Wakayashi et al., 1988; 1989 (as evidenced by the presence of Lewy bodies)
(5b) Singaram et al., 1995
(5c) Wakabayashi et al., 1990 (as evidenced by immunohistochemistry)
(5d) Foley and Riederer, 2000
(6) Buchan, 1990
(7) Meneghelli, 1985
(7b) Ribiero et al., 1998

autosomal dominant, slowly progressive disorder of unknown pathogenesis whose characteristic feature is myotonia (Harper, 1979), gastrointestinal motility disturbances are common (Nowak et al., 1982). Although in some cases, the smooth muscle is the indicated site of the gastrointestinal pathology (Pruzanksi and Huvos, 1967; Keschner and Davison, 1933), in others it consists of a degenerative neuropathy of the myenteric plexus (Yoshida et al., 1988). Damage to enteric neurons has also been noted in diabetes mellitis, in which the body has dysfunctional glucose metabolism due either to impaired insulin production or to the body's resistance to insulin actions (Buchan, 1990). GI motility disorders are also the most frequent autonomic disorders in patients with Parkinson's disease, and may occur before the first symptoms of Parkinsonism appear. Although the etiology of Parkinson's disease is not known, evidence implicates oxidative stress processes in the neuropathogenesis of this disease (Foley and Riederer, 2000). Finally, although the underlying pathogenesis of enteric neurodegeneration in Chagas' disease (*Trypanosoma cruzi* infection) is unknown, severe damage to enteric neurons throughout the digestive tract is commonly noted in this disease. Indeed, cardiac disease and digestive abnormalities (thought to result from ENS damage) are the primary symptoms of chronic Chagas' disease (Meneghelli, 1985).

While ENS damage and/or degeneration can occur within a number of diseases, ENS damage does not display consistent characteristics between diseases. The specific neural plexuses that are damaged, the nature of nerve fibre alterations that occur, those neurochemical subsets of enteric neurons that are either selectively damaged or preferentially spared, and specific increases or decreases in the expression of certain enteric neurotransmitters, all vary between different diseases (see Table 2). For example,

whereas both the myenteric and submucosal plexuses are affected in IBD (Riemann and Schmidt, 1982; Dvorak et al., 1980; Steinhoff et al., 1988; Lindgren et al., 1991), damage is confined exclusively to the myenteric plexus in chronic intestinal pseudo-obstruction (Chinn and Schuffler, 1988). While in IBD, both axonal necrosis (Dvorak and Silen, 1985; Steinhoff et al., 1988; Kubota et al., 1992) and axonal hyperplasia (Leonard et al., 1995; Strobach et al., 1990) occur, axonal degeneration and loss characterize chronic intestinal pseudoobstruction (Chin and Schuffler, 1988). Selective sparing of VIP- and NPY-positive neurons and preferential damage of Substance P- and enkephalin-positive neurons is characteristic of myotonic dystrophy (Yoshida et al., 1988); contrasting with the selective sparing of ACh-positive neurons of the myenteric plexus and the preferential damage to CGRP- and somatostatin-positive neurons of the submucosal plexus characteristic of diabetes mellitis (Buchan, 1990). These marked differences in ENS damage characteristics suggest both that specific ENS damage characteristics may be representative of given diseases, and that clinical enteric neurodegeneration may occur via several different damage mechanisms.

1.3.5 Experimental Study of Enteric Neurodegeneration

The correlation between the clinical occurrence of intestinal inflammation and enteric nervous system damage is striking. However, experimental studies are required to establish that enteric neurodegeneration occurs as a consequence of inflammation and to identify those specific mediators of the inflammatory process which induce neurodegeneration. Much of the existing data on neurodegenerative mechanisms has been generated using neuronal cell lines or primary neuronal preparations from outside

the gut (Hall and Wiley, 1998). To date, very few studies have examined the direct effects of inflammation or specific inflammatory mediators on ENS viability.

Animal models of intestinal inflammation have provided the opportunity to directly investigate the impact of inflammatory processes on the ENS (Jacobson et al., 1995). Several animal models of inflammation are commonly used, including chemical-induced inflammation, which is a simple and affordable procedure hindered only by its lack of chronicity (Elson et al., 1995). Immunologically-mediated inflammation is also commonly induced by trinitrobenzene-sulfonic acid (TNBS), which when attached to a carrier protein, elicits the formation of antibodies. However, this model suffers from a high variability in the induced inflammatory response (Elson et al., 1995). Finally, infection-induced inflammation is frequently induced using the nematode *Trichinella spiralis* (Grossi et al., 1993; Jacobson et al., 1995). In all of these models, tissue responses must be shown to result from the inflammation itself, and not from a direct toxic effect of the inflammatory initiator. To this end, attenuation or prevention of tissue responses by anti-inflammatory treatment is commonly used to elucidate the role of inflammatory processes in tissue damage. By such means, it has been determined that ENS functional alterations, damage and degeneration occur as a consequence of intestinal inflammation itself, and are independent of the manner in which inflammation is induced (Jacobson et al., 1995; Collins, 1996; Palmen et al., 1998; Sanovic et al., 1999). Furthermore, correlations between specific inflammatory cell types and ENS damage have been evaluated in a DNBS-induced model of intestinal inflammation (Sanovic et al., 1999). In this animal model, early inflammatory events were suggested to induce neuronal loss. The presence of eosinophils within and adjacent to the myenteric ganglion

corresponded with the occurrence of neuronal damage within these enteric neurons, suggesting that eosinophils may be responsible for neuronal damage and loss.

Recent studies have begun to investigate functional disturbances of the enteric nervous system that correlate with observed structural damage during intestinal inflammation. In animal models of intestinal inflammation, evoked neurotransmission was markedly suppressed within the enteric nervous system, independent of the manner in which inflammation was induced (Jacobson et al., 1995). These functional alterations were not restricted to inflamed areas of intestine, but also occurred within noninflamed bowel segments. These functional observations correlate with the clinical findings of ultrastructural changes in enteric nerves from noninflamed bowel segments of Crohn's disease patients (Dvorak et al., 1980), and with observed intestinal transit and motor changes from noninflamed gut regions of ulcerative colitis patients (Rao et al., 1987; Manousos and Salem, 1965). While these experimental and clinical findings do not dispute the notion that inflammation induces ENS damage, they suggest that inflammation-associated alterations in the enteric nervous system are not restricted to the sites of overt inflammation. These functional and structural changes observed in non inflamed regions are thought to reflect some unknown systemic component of the inflammatory process (Jacobson et al., 1995). Thus, inflammatory damage mechanisms in the enteric nervous system may be complex; comprising both localized and systemic components.

1.3.6 Putative Mediators of Enteric Neurodegeneration

To date, sparse information exists regarding the identity of specific mediators and mechanisms of enteric neuron death. A small number of candidate compounds, identified

to act in CNS neurodegeneration are likely to participate in inflammatory ENS damage. Within the ENS, as elsewhere, nitric oxide (NO) is a mediator of inflammatory neurodegeneration and more general cell death. N^G-nitro-L-arginine methyl ester (L-NAME)-induced inhibition of the high concentrations of NO produced during experimental inflammation attenuates the induction of marked morphological disruptions of myenteric ganglia and suppression of myenteric nerve function observed *in vivo* (Hogaboam et al., 1995). These findings support a role for NO in enteric neurodegeneration. However, it is not known if NO directly induces this neurodegeneration. While NO may directly damage the ENS, NO interactions with other cell types within the intestinal wall could indirectly induce ENS damage, either through the production of toxic products, or through inhibition of neuroprotection or trophic support.

Proinflammatory cytokines have also been implicated in the inflammatory pathogenesis of enteric neurons. Platelet-activating factor (PAF), a mediator of the proinflammatory cytokine TNF (Im et al., 1996; Carden and Granger, 2000), rapidly induces cell death in a subset of cultured neonatal myenteric plexus neurons (Willard, 1992). However, the biological significance of these findings remains to be determined. While the expression of the proinflammatory cytokines interleukin-1β (IL-1β) and interleukin-6 (IL-6) is increased during inflammation, and their actions alter neurotransmitter release from enteric neurons, there is as yet no evidence that these molecules are directly involved in enteric neuronal death (Khan et al., 1992; Khan and Collins, 1994; Main et al., 1993; Xia et al., 1999).

Finally, the excitatory neurotransmitter glutamate has been implicated in the excitotoxic damage and degeneration of enteric neurons (Kirchgessner et al., 1997). This neurotransmitter is considered the chemical inducer of hypoxic and ischemic damage (Bouvier et al., 1992), and reactive oxygen species are thought to mediate much of the intracellular injury evoked by glutamate (Bonfoco et al., 1996). The evidence implicating glutamate in ENS damage is compelling; glutamate and its agonist, *N*-methyl-d-aspartate (NMDA), induce comparable neuronal cell death in both intact preparations of bowel and in cell cultures of adult myenteric neurons. The delayed neuronal death induced within these enteric neurons is prevented by the NMDA antagonist D-2-amino-5-phosphonopentanoate. As in other systems in which glutamate-induced excitotoxicity occurs (Marc et al., 1990; van den Pol, 1991), glutamate, glutamate receptors, and high-affinity glutamate transporters are all localized to ENS neurons (Kirchgessner et al., 1997). From these findings, one can conclude that glutamate is likely a mediator of enteric neurodegeneration.

The AMPA and kainate types of glutamate receptor may also factor into the axonal injury characteristic of clinical ENS damage. In the CNS, NMDA receptors are primarily implicated in CNS excitotoxicity, and AMPA and kainic acid receptors are involved in axonal injury, which is independent of synaptic activity and the neurotransmitter actions of glutamate (Li and Stys, 2000). In spinal cord white matter anoxic and traumatic injury, Na^+-dependent glutamate transport is reversed, activating AMPA receptors and thus releasing toxic levels of endogenous glutamate (Li et al., 1999).

Although the study of enteric neurodegeneration is in its infancy, with very little known about neurotoxic actions of specific inflammatory mediators, experimental models of inflammation have established a causal relationship between intestinal inflammation and enteric nerve damage, and this has permitted some characterization of the factors involved. The recent use of both enteric cell cultures and *in vivo* models of gut diseases has greatly contributed to such investigations. Indeed, cytokines, excitotoxicity, and reactive oxygen species, all of which are known inflammatory mediators of neurodegeneration within the CNS (Martiney et al., 1998), have been established as likely candidates to induce neurodegeneration within the gut.

1.4 Inflammation and associated processes

1.4.1 Inflammation

Tissue injury caused by pathogen infection or physical damage evokes inflammation, a physiological immune defense reaction to an injurious stimulus (Vaday and Lider, 2000). The immune system is responsible for the clearance of viruses, bacteria, foreign proteins, and malignant cells from the body. Inflammatory responses are the rapid responses of the immune system which eliminate, control or contain an injurious stimulus and ensuing cellular damage (Martiney et al., 1998). The ultimate function of inflammation is to restore tissue homeostasis (Viday and Lider, 2000). Regardless of the nature of the inflammatory stimulus, a standardized sequence of events ensues whereby the injurious agent is localized and destroyed, and damaged tissue repaired (see Figure 4).

Inflammatory responses are initiated immediately following the detection of trauma or invading pathogens by leukocytes. Leukocytes are found within the body's

circulatory system, and are the principle cells involved in inflammatory immune responses. Leukocytes consist of granulocytes and macrophages, which mount generalized responses to all injurious stimuli, and lymphocytes, which are specialized to attack specific injurious stimuli. Granulocytes (which comprise neutrophils, eosinophils and basophils) release cytotoxic compounds from their intracellular granules into the local environment when they encounter microorganisms or malignant cells. The ensuing cellular destruction occurs rapidly, and may harm healthy tissue of the body in addition to infected cells and foreign materials.

Macrophages, the other class of leukocytic defence cells from the non-specific immune system, can consume microorganisms and malignant cells by phagocytosis or, similar to granulocytes, by the secretion of cytotoxic compounds. However, macrophages can also act more specifically by collaborating with lymphocytes and their products.

The lymphoid system, the specific immune defence, is represented by two cell types: B-lymphocytes which produce antibodies that bind to foreign organisms and facilitate their destruction by activating the complement system, and T-lymphocytes which act mainly by cell-to-cell contact. One subpopulation of T-lymphocytes (killer T-cells) recognizes and kills cells that bear foreign antigen (e.g. following virus infection). The second population (helper T-cells) modulates the activity of other leukocytes (Imhof and Dunon, 1997). The specific nature of the injurious stimuli determines that type of leukocytes involved in the inflammatory response.

Upon interaction of circulating leukocytes with pathogens or malignant cells, leukocytes become activated and release pro-inflammatory cytokines; initiating

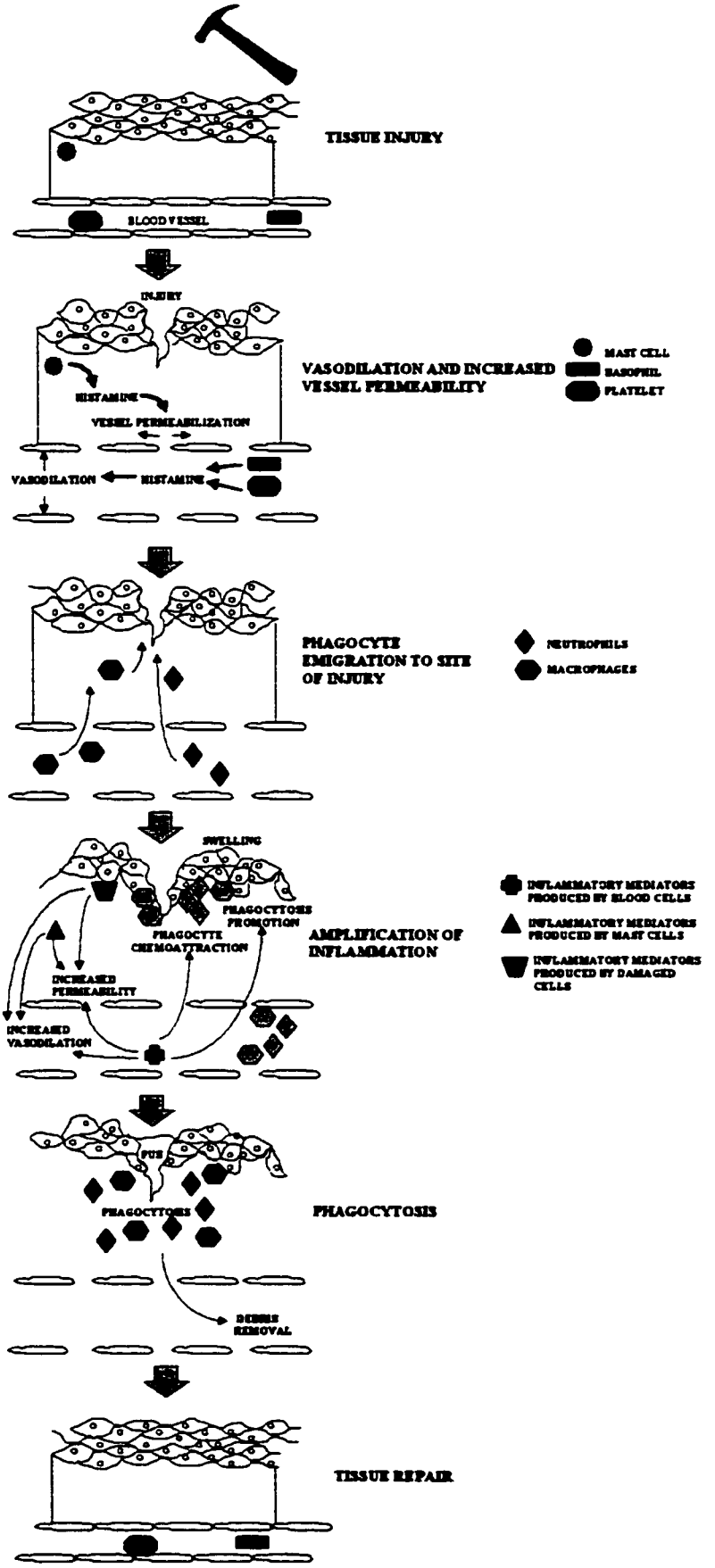

TISSUE INJURY
BLOOD VESSEL
INJURY
HISTAMINE
VESSEL PERMEABILIZATION
VASODILATION
HISTAMINE
VASODILATION AND INCREASED VESSEL PERMEABILITY
MAST CELL
BASOPHIL
PLATELET
PHAGOCYTE EMIGRATION TO SITE OF INJURY
NEUTROPHILS
MACROPHAGES
SWELLING
PHAGOCYTOSIS PROMOTION
PHAGOCYTE CHEMOATTRACTION
INCREASED PERMEABILITY
INCREASED VASODILATION
AMPLIFICATION OF INFLAMMATION
INFLAMMATORY MEDIATORS PRODUCED BY BLOOD CELLS
INFLAMMATORY MEDIATORS PRODUCED BY MAST CELLS
INFLAMMATORY MEDIATORS PRODUCED BY DAMAGED CELLS
PUS
PHAGOCYTOSIS
DEBRIS REMOVAL
TISSUE REPAIR

Figure 4. Schematic representation of a generalized inflammatory reaction. Following an injurious stimulus, mast cells in the injured tissue and basophils and platelets within blood vessels release histamine and kinins, increasing blood vessel permeability and stimulating phagocyte emigration to the site of injury. Blood cells, mast cells, and damaged cells produce inflammatory mediators that amplify inflammation by increasing vasodilation and vessel permeability, attracting circulating immune cells, and promoting phagocytosis of damaged cells and/or invading microorganisms. Cellular debris is then removed through the circulatory system and pus fills the site of injury. As the injury is contained and tissue damage repaired, the inflammatory response subsides.

inflammatory responses. Cytokines, a family of polypeptides produced mainly by activated leukocytes, interact to initiate, propagate, and then ultimately suppress inflammation. An important contribution of the pro-inflammatory cytokine cascade is the induction of an inflammatory response that recruits and activates subsets of leukocytes that mediate much of the response to the sensitizing antigen. These proinflammatory cytokines also activate endothelial cells to express adhesion molecules and induce the release of chemokines (proteins involved in chemoattraction); thus focusing and directing the inflammatory response to the injury site (Martiney et al., 1998).

The expression of adhesion molecules is induced by pro-inflammatory cytokines, and is necessary for the binding and transmigration of leukocytes from the blood, across the vasculature, to sites of injury and inflammation (Martiney et al., 1998). ICAM-1, VCAM-1, and selectins are endothelial cell adhesion molecules that slow leukocyte passage through blood vessels; thus facilitating the interaction and binding of leukocytes to the vessel endothelium (Imhof and Dunon, 1997). Such binding occurs via the interaction of endothelial adhesion molecules with integrins, adhesion molecules located on the surface of leukocytes (Lloyd et al., 1996). Integrins assist in moving these leukocytes through the blood vessel wall and into the interstitial matrix.

Pro-inflammatory cytokines, such as IL-1 and TNF, also induce the expression of chemokines - small proteins, secreted primarily by vessel endothelium, that activate integrins and the chemotactic migration of circulating leukocytes to the site of inflammation (Martiney et al., 1998; Baggiolini et al., 1994). Chemokines impose directionality on the inflammatory process, stabilizing leukocyte interactions with adhesion molecules and thus preparing these cells for migration from the

microvasculature into the interstitial matrix. These proteins also activate and attract specific subsets of leukocytes to the inflammatory site (Taub and Oppenheim, 1994; Glabinski et al., 1995).

Vessel dilation and endothelial cell contraction also facilitate the migration of accumulated leukocytes into the inflammation site by increasing vessel permeability to these cells. The early release of histamine by damaged cells at the site of injury, mast cells in connective tissue, and basophils and platelets in blood, induces blood vessel vasodilation, and increases blood vessel permeability. Kinins, formed in blood by proteases released from damaged cells, perform similar functions. These molecules also act as chemotactic agents for phagocytic leukocyte migration. The effects of histamine and kinins are intensified by prostaglandins released by damaged cells, and leukotrienes produced by activated basophils and mast cells. Finally, the complement system of serum proteins, activated by antibody-antigen complexes and microorganisms, destroys microorganisms and enhances the destructive abilities of phagocytic cells. Biologically active protein fragments generated during the proteolytic complement cascade stimulate mast cells and basophils to secrete histamine. The histamine increases the permeability of local blood vessels, allowing leukocytes, antibodies and complement to enter the site of infection (Eckert et al., 1988).

The result of these complex inflammatory changes is that blood vessels dilate and endothelial cells of the microvasculature adhere less tightly to one another, but their surfaces adhere more avidly to passing leukocytes. Leukocytes, attracted to the inflammatory site, are caught and extravasated by passage between the endothelial cells. This extravasation process, which permits access to the injury site where invading

pathogens and malignant cells can be destroyed, is central to the establishment of inflammation (Vaday and Lider, 2000).

With facilitated extravasation, the inflammatory reaction expands dramatically (Sartor, 1995). The inflammation site attracts more leukocytes, producing a self-amplifying inflammatory cycle. Neutrophils, followed by macrophages, emigrate through the permeabilized microvasculature into injured tissue, where cellular debris, microorganisms, and other particles are phagocytized. Anti-inflammatory cytokines, including TGF-β, IL-10, and IL-4, signal down-regulation of inflammation as damage is repaired. These cytokines deactivate endothelial cells and macrophages, inhibit lymphocyte proliferation and reduce leukocyte adhesion (Wahl, 1994).

While the inflammatory response destroys injurious stimuli within the body, the toxic nature of many of its byproducts can cause significant damage to adjacent tissues (Ward, 1991; Birkedal-Hansen, 1993). A high probability of tissue damage exists when inflammation is chronic, as in several disease states. At inflammatory sites, blood flow-rate is reduced by the loss of intravascular fluid and by increases in plasma viscosity. Although this vascular stasis promotes leukocyte adhesion to the endothelium and facilitates their migration to the interstitial matrix, reduced blood flow can also induce damaging hypoxia or ischemia in surrounding tissues (Imhof and Dunon, 1997). Activated leukocytes release a wide variety of lytic enzymes as they migrate through the extracellular matrix, as well as reactive oxygen and nitrogen intermediaries following phagocytosis of bacteria and tissue debris, that could also damage adjacent tissue. Finally, cytokine release at the site of inflammation often has significant cytotoxic effects on surrounding tissues (Vassalli, 1992). Enteric neurons are subject to these potential

damage mechanisms during inflammatory diseases of the gut, and this toxicity may underlie the degeneration of these cells under these conditions. In our studies, we thus examined the role of potentially toxic mediators of inflammation on the viability of enteric neurons.

1.4.2 Hypoxia-reoxygenation

When blood flow is disrupted, the constant supply of oxygen and glucose to cells is impaired. Cells may experience hypoxia, in which oxygen levels are reduced or, with prolonged or extensive blood flow disruption, cells may experience ischemia, in which glucose levels as well as oxygen levels are reduced. In both hypoxia and ischemia, affected cells exhibit profound decreases in the levels of adenosine triphosphate (ATP), triggering multiple degenerative processes. During hypoxic insult, anaerobic glycolysis, although an inefficient reaction in neurons, may proceed when aerobic processes cannot in order to supply ATP for cellular energy requirements. During ischemia, however, both glucose depletion and oxygen depletion prevent anaerobic glycolysis from proceeding; causing more rapid and severe cell damage (Saikumar et al., 1998).

It is obvious that if blood flow is not restored to hypoxic tissues, they will die. However, if blood flow is restored prior to cell death, a second stage of tissue injury ensues. This is reperfusion injury, which paradoxically produces tissue damage because of the actions of oxygen in energy-depleted tissue (Mishra and Delivoria-Papadopoulos, 1999; Saikumar et al., 1998). Hypoxia-reoxygenation injury can be divided into three general stages: 1) initial hypoxic events characterized by rapidly developing bioenergetic failure and loss of ion homeostasis; 2) reoxygenation, where the bioenergetic state is restored and synaptic activity returns; and 3) final secondary cell death (Kristian and

Siesjo, 1996). A multitude of interconnecting damage mechanisms are thought to underlie hypoxia-reoxygenation injury including decreases in intracellular energy and pH, mitochondrial damage and ATP depletion, release of excitatory amino acids, excessive NMDA receptor activation, increases in intracellular Ca^{2+} ions, and the production of reactive oxygen species (Mishra and Delivoria-Papadopoulos, 1999).

During hypoxic insult, oxygen supplies are rapidly depleted by their use in glycolytic energy production, and neurons must then switch to anaerobic glycolysis to meet their energy requirements. During anaerobic glycolysis, HCO_3- levels decrease and CO_2 levels increase, inducing rapid decreases in intracellular pH to reach acidic levels (Tombaugh and Sapolsky, 1993). Acidotic injury is one of the oldest theories proposed to explain hypoxic damage, but the precise mechanisms whereby acidosis damages nervous tissue remain unclear (Budd, 1998).

With continued hypoxia ATP levels fall (Kraig et al., 1985) and Na^+/K^+-ATPase activity fails, leading to a rise in extracellular K^+ and intracellular Na^+ concentrations termed anoxic depolarization (Hansen, 1985). In response, Cl- and H_2O enter the cell to preserve electrical neutrality and intracellular osmolarity (Choi, 1990; Siesjo et al., 1991). This H_2O influx results in neuronal swelling, which is associated with abnormalities in membrane permeability and may be lethal to neurons (Maulucci-Gedde and Choi, 1987; Choi, 1987). The high intracellular Na^+ levels of anoxic depolarization stimulate ATP-dependent Na^+ extrusion, precipitating declines in already-depleted cellular ATP reserves (Marcaida et al., 1996). High intracellular Na^+ levels reverse the direction of both Na^+-dependent glutamate transporters and Na^+/ Ca^{2+} exchangers (Yamaguchi et al., 1998;

Khodorov et al., 1993; White and Reynolds, 1995), thus contributing to high levels of intracellular Ca^{2+} that induce neuronal damage (White and Reynolds, 1997).

During hypoxia, early membrane damage activates multiple degradative systems to induce primarily necrotic death (Leist et al., 1997). Cells subjected to sublethal hypoxia can be damaged during subsequent reoxygenation injury. Reoxygenation can damage cells and induce cell death even though, and in fact because, cells restore ATP pools depleted during hypoxia (Eguchi et al., 1997; Leist et al., 1997). Apoptotic and necrotic cell death mechanisms may also occur simultaneously in cells exposed to hypoxia-reoxygenation, with the intensity and duration of insult determining the cell death mechanisms involved (Bonfoco et al., 1995; Leist and Nicotera, 1997).

The gut is predisposed towards damage by hypoxia and ischemia due to its unique vascular anatomy in which blood flows through intestinal villi that are highly susceptible to injury (Taylor, 1998). Gut hypoxia/ischemia can also itself promote local inflammation (Massberg et al., 1998). Hypoxia/ischemia promotes regional production of inflammatory mediators, expression of cell adhesion molecules on endothelial and immune cell surfaces, and increases the procoagulatory properties of vascular endothelial cells (Schwarz et al., 1999). During reoxygenation/reperfusion, increased concentrations of oxyradicals are produced that indirectly trigger the accumulation of neutrophils within affected tissues and promote inflammatory processes (Schoenberg and Beger, 1993). Hypoxia/ischemia is also a frequent consequence of gut inflammation during diseases including IBD in which blood flow can be compromised during severe tissue inflammation (MacCannell, 1993). Enteric neurons are likely damaged during such tissue inflammation by hypoxic/ischemic insults. Although no studies of hypoxic

damage in the ENS have been performed, such damage would presumably occur via mechanisms similar to those underlying CNS hypoxic damage. The experimental study of hypoxic insult on the viability of enteric neurons is presented in **Chapter 2** of this thesis.

1.4.3 Cytotoxicity

Investigation of hypoxic and ischemic injury has generated extensive literature on cytotoxicity. Indeed, intracellular damage mechanisms elucidated through study of hypoxia and ischemia are representative of intracellular responses to cytotoxic challenge in general.

Increases in intracellular Ca^{2+} concentrations are fundamental to cytotoxic processes (Choi, 1995; Kristian and Siesjo, 1998). Intracellular Ca^{2+} concentrations are commonly increased via intracellular release mechanisms (Frandsen and Schousboe, 1993), Ca^{2+} entry through voltage-activated Ca^{2+} channels, or Ca^{2+} entry through agonist-activated Ca^{2+} channels (Choi, 1990). Intracellular accumulation of Na^{+}, inositol 1,4,5-triphosphate (IP_3), and diacylglycerol (DAG), induced by cytotoxic challenge, can also elevate intracellular Ca^{2+} concentrations and contribute to cellular damage (Choi, 1990; Masu et al., 1991; Kiedrowski et al., 1994). Elevated levels of Ca^{2+} within cells readily bind calmodulin and so activate nitric oxide synthase (NOS), generating toxic levels of nitric oxide, peroxynitrite, and oxygen free radicals that impair cellular and subcellular membrane integrity (Bonfoco et al., 1996). High Ca^{2+} concentrations can also activate phospholipase A_2, releasing arachidonic acid which, when metabolized by cyclooxygenase and lipoxygenase, produces toxic oxygen free radicals (Lazarewicz et al., 1990).

Increased intracellular Ca^{2+} levels may also lead to elevated intranuclear Ca^{2+} levels through activation of high affinity Ca^{2+}-ATPase transporters, IP_4 receptors, or IP_3 receptors located on the nuclear membrane (Humbert et al., 1996; Karin, 1992). Nuclear Ca^{2+} controls a number of critical nuclear functions including regulation of transcription factors, cell cycle regulation, gene transcription, DNA replication and nuclear envelope breakdown, and thus potentially controls complex nuclear events, leading to hypoxia-induced cell death (Karin, 1992; Steinhardt and Alderton, 1988; Tombes et al., 1992).

Cytoplasmic Ca^{2+} overload has also been implicated in cytotoxicity resulting from mitochondrial damage (Isaev et al., 1996). In general, mitochondria buffer high intracellular Ca^{2+} concentrations (Herrington et al., 1996). However, the accumulation of large amounts of Ca^{2+} intracellularly, as during hypoxic insult, can precipitate catastrophic changes in mitochondrial permeability, initiating apoptotic cascades and leading to cell death (Isaev et al., 1996; Atlante et al., 1996).

Oxygen free radicals, highly reactive molecules with one or more unpaired electrons in their outer orbital, mediate much intracellular damage (Schoenberg and Beger, 1993). These molecules, generated by a number of Ca^{2+}-dependent reactions, can damage proteins, lipids and DNA, and impair membrane integrity and alter ion channel activity (Grisham, 1994; Larramendy et al., 1987; Evans, 1993; Esterbauer et al., 1991). Once formed, free radicals may also promote excitotoxic injury by facilitating glutamate release (Pellegrini-Giampietro et al., 1988).

Calpain, an Ca^{2+}-activated neutral cysteine protease, also plays an important role in cellular damage mechanisms (Bartus, 1997; Markgraf et al., 1998). Once activated by increased intracellular Ca^{2+} concentrations, this enzyme damages critical cytoskeletal

elements via its proteolytic cleavage of spectrin, tubulin, and ankyrin (Saido et al., 1994). Calpain also damages cells by opening the mitochondrial permeability transition pore, initiating apoptotic cascades (Aguilar et al., 1996).

Mitochondrial damage has also been implicated in cytotoxic mechanisms of action. Mitochondrial membrane damage induces electron transport defects, impaired energy production, and eventual cell death (Piper et al., 1994). High intracellular Ca^{2+} concentrations, oxidative stress, and reductions in mitochondrial membrane potential can induce mitochondrial permeability transition pore (PTP) opening (Zoratti and Szabo, 1995). Prolonged PTP opening permits diffusion of solutes less than 1500 daltons in size between the inner and outer mitochondrial membranes (Frade and Michaeldis, 1997), and can damage neurons by inducing mitochondrial swelling and membrane rupture (Petit et al., 1998; Reed et al., 1998). Even in the absence of mitochondrial destruction, PTP opening can induce apoptotic cell death by release of the protein apoptosis inducing factor (AIF) into the cytosol (Zamzami et al., 1995 a, b). AIF is thought to have direct proteolytic activity and to directly initiate cellular apoptosis (Susin et al., 1996). Release of cytochrome c, an integral component of the electron transport chain required for respiration and oxidative phosphorylation (Saikumar et al., 1998), from the intermembraneous space during PTP opening has also been proposed to mediate mitochondrially-induced apoptosis (Liu et al., 1996).

Thus, cellular insult and injury can induce multiple intracellular pathways leading to cytotoxic cell death. Impaired cellular homeostasis, as commonly manifested by altered Ca^{2+} concentrations, generally initiates such damage pathways. Resultant protein

and DNA damage, membrane disruption and mitochondrial dysfunction can all contribute to cellular toxicity.

1.5 Inflammatory mediators

1.5.1 Reactive Oxygen Species

Reactive oxygen species (ROS) are oxygen-based free radicals that play important roles in inflammatory tissue damage. A free radical is defined as an atom or molecule that contains one or more unpaired electrons. ROS include the superoxide molecule ($O_2^{\cdot}$-), peroxide radical ($O_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$), nitric oxide ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$), and peroxynitrite ($ONOO^{\cdot}$-) (Anderson et al., 1999).

Under normal conditions, ROS act as intracellular signaling molecules, activating the highly ubiquitous transcription factors activator protein-1 (AP-1) and nuclear factor kappa B (NFκB), which in turn activate a large number of genes involved in cell growth and early cellular defence reactions (Pahl and Baeuerle, 1994). ROS are uniquely suited for this signaling molecule role, as they are easily synthesized and degraded, highly diffusible, and ubiquitous within all types of cells (Remacle et al., 1995; Suzuki et al., 1997). However, these molecules can also damage cellular components in a process termed oxidative stress (Pauling, 1964) (see Figure 5). Due to the ubiquitous use of ROS, cells are under constant oxidative stress which, while required for survival and generally kept in check by endogenous anti-oxidant defence systems, can cumulatively damage cells and so contribute to a variety of chronic disorders (Cross et al., 1987). ROS production also increases dramatically during inflammatory processes, during which ROS can acutely damage surrounding tissues.

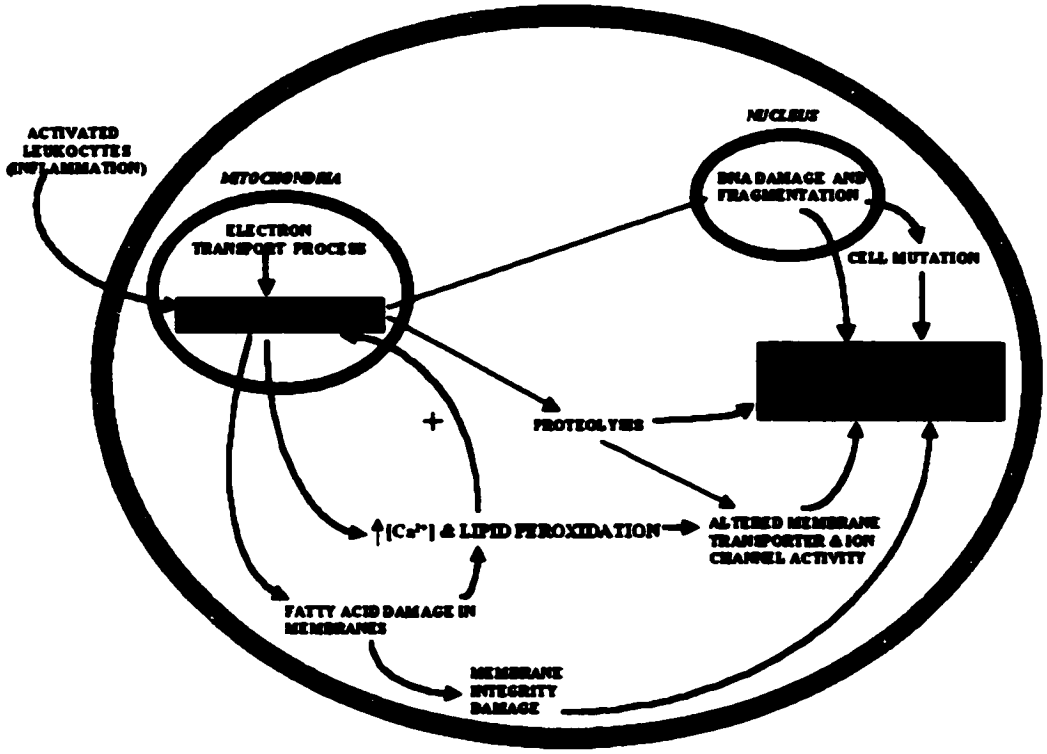

ACTIVATED LEUKOCYTES (INFLAMMATION)
MITOCHONDRIA
ELECTRON TRANSPORT PROCESS
NUCLEUS
DNA DAMAGE AND FRAGMENTATION
CELL MUTATION
+
PROTEOLYSES
↑[Ca²⁺] & LIPID PEROXIDATION
ALTERED MEMBRANE TRANSPORTER & ION CHANNEL ACTIVITY
FATTY ACID DAMAGE IN MEMBRANES
MEMBRANE INTEGRITY DAMAGE

Figure 5. Cytotoxic pathways of reactive oxygen species (ROS). The pre-eminent subcellular source of ROS is the electron transport process within mitochondria. During inflammation, ROS production is increased dramatically both intracellularly and, in particular, within activated leukocytes. ROS have high reactivity and can thus directly interact with and damage fatty acids within membranes, DNA within cell nuclei, and induce proteolysis and lipid peroxidation. Such intracellular damage can deplete cellular energy sources and lead to cell death. In addition to intracellular damage, ROS released into the extracellular matrix can induce the migration of activated immune cells, which actively phagocytose susceptible cells.

The biochemical properties of different ROS members vary widely, and so determine their roles in both normal cellular function and in damage mechanisms. $OH^{\cdot}$, the hydroxyl radical, is an extremely reactive molecule which cannot diffuse more than one or two molecular diameters before reacting with a cellular component (Pryor, 1986). Thus, its actions are highly localized. In contrast to $OH^{\cdot}$, ONOO-, the peroxynitrite radical, can diffuse within cells and may be taken up by adjacent cells via anion transporters (Radi, 1998); accounting for its widespread actions. Due to its relatively low reactivity, O_2- is also able to diffuse intact some distance before reacting with other molecules (Braughler and Hall, 1989). Although O_2- is itself relatively innocuous, it can react with a wide variety of molecules to form several highly reactive oxygen radicals, including alkyl- ($RO^{\cdot}$) and peroxyl- ($ROO^{\cdot}$) radicals, nitrogen radicals such as peroxynitrite ($ONOO^{\cdot}$-), and oxyhalides including hypochlorous acid (HOCl) (Bergendi et al., 1999).

ROS are produced by several intracellular systems during normal cellular functions. These sources of ROS include plasma membrane NAD/NADPH systems, microsomal cytochrome P450, and peroxisomes. However, in most eukaryotes, mitochondria are the major sources of ROS, generating single electrons during electron transport which partially reduce O_2, to $O_2^{\cdot}$. (Anderson et al., 1999; Powis et al., 1995).

Additional sources of ROS manifest during inflammation and associated processes. During traumatic injury or inflammation, arachidonic acid is released within cells as a consequence of lipid peroxidation, elevated Ca^{2+} concentrations, or cellular activation (Siesjo, 1981; Bazan, 1976; Sevanian et al., 1985; van Kuijk et al., 1987). Metabolism of arachidonic acid by the cyclooxygenase pathway directly forms toxic

ROS compounds (Yamamoto, 1983; Kukreja et al., 1986). Alterations in cellular metabolism, which are a common result of hypoxia and ischemia, induce uncoupling of electron transport chain components, dramatically increasing electron leakage and ROS production (Patole et al., 1986; Braugler and Hall, 1989). Finally, xanthine oxidase, also upregulated during hypoxia, readily degrades O_2 re-introduced during reoxygenation to form O_2- and H_2O_2, which readily damages surrounding tissue (McCord et al., 1968; McCord, 1985).

During inflammation, respiratory bursts are triggered in neutrophils and macrophages (Bergendi et al., 1999) such that NADPH oxidase complexes localized to the cell membrane reduce O_2 to $O_2^{\cdot}$- (oxygen to superoxide). $O_2^{\cdot}$- released into the extracellular matrix can, by itself or by formation of other ROS members, attack infectious agents, foreign particles and damaged tissue within the inflammatory site (Grisham and Granger, 1988). However, ROS are indiscriminate in their actions; reacting with the nearest available molecule. Thus, the localized high concentrations of ROS produced during inflammation can also induce significant damage to host tissues at inflammatory sites (McCord, 1987).

Iron is extremely important within ROS-induced damage of inflammation and associated ischemic processes. Under normal circumstances, redox active iron is maintained at extremely low levels both outside and within cells. Extracellularly, the iron transport protein transferrin binds iron. Intracellularly, iron is sequestered by the iron storage protein ferritin. During ischemia, tissue pH declines to levels at which both transferrin and ferritin release iron (Gutteridge, 1986; Thomas et al., 1985). Once released, free iron (Fe^{++}) can react with O_2 to form $O_2^{\cdot}$- or with H_2O_2 to form $OH^{\cdot}$ or

FeOH, both of which are extremely potent oxidants. Thus, the presence of iron can greatly enhance ROS formation and toxicity (Braughler and Hall, 1989).

ROS attack and damage a wide range of target molecules within cells. ROS can directly damage DNA bases and induce DNA strand breaks by interaction with deoxyribose residues (Ames, 1989). ROS can also damage DNA indirectly, via oxidization of lipid or protein molecules to generate intermediates that form adducts upon reaction with DNA (Ward et al., 1987). Polyunsaturated fatty acid residues of membrane phospholipids can also be modified by ROS actions, compromising membrane integrity and producing toxic lipid peroxidation products (as detailed in *Section 1.5.3, 4-hydroxy-2-nonenal*) (Fink et al., 1997; Sodum and Chung, 1988). Finally, functional groups on proteins, including sulphhydryl groups involved in the regulation of protein function, can interact with ROS (Powis et al., 1995; Sen and Packer, 1996; Droge et al., 1992). By these means, many enzymes including protein kinases and phosphatases can be functionally altered, protein binding to other macromolecules such as DNA can be impaired, and homeostatic mechanisms for maintaining cytosolic Ca^{2+} concentrations can be modified by disruption of membrane transport protein function (McConkey and Orrenius, 1996).

Thus, ROS play an important role in inflammatory tissue damage and have widespread toxicity, being generated by multiple pathways during inflammation and reacting with a wide variety of intracellular targets to effect damage. High levels of ROS have been noted within intestinal tissues during inflammation, and have been implicated in the pathogenesis of intestinal inflammation and disease (Keshavarzian et al., 1992; Simmonds et al., 1992; Oshitani et al., 1993). ROS have also been implicated in neuronal

damage within the CNS (Jesberger and Richardson, 1991; Mattson et al., 1996; Simonian and Coyle, 1996). Given the involvement of ROS in CNS neurodegeneration and in inflammatory intestinal damage, these molecules are considered likely candidates to effect enteric neuron damage during intestinal inflammation. Investigation of the roles of specific ROS in enteric neurodegeneration is presented in **Chapter 2** of this thesis.

1.5.2 Nitric Oxide

The reactive oxygen species, nitric oxide (NO) is a ROS member of particular interest, as it has specialized functions, distinct from those of other ROS during both normal cellular events and within inflammation.

Nitric oxide (NO) is a colorless gas containing an odd number of electrons, and is thus a free radical by definition. NO is known as a common air pollutant, toxic gas, and constituent of cigarette smoke. Within vertebrate cells, NO serves several different functions. It is responsible for the non-adrenergic, non-cholinergic vasodilation of blood vessels (Moncada et al., 1989; 1991; Ignarro, 1990), mediates macrophage cytotoxicity (Marletta, 1989), and is a putative inhibitory neurotransmitter in both the central and peripheral nervous systems (Snyder and Bredt, 1991; Garthwaite, 1991). Conversely, in situations of excessive production, this neurotransmitter may also act as a neurotoxin (Dawson et al., 1991) and is produced specifically as a anti-microbial agent and cytotoxin by activated macrophages (Beckman et al., 1990; Beckman and Crow, 1993). Unlike conventional neurotransmitters, NO is not stored in synaptic vesicles nor does it undergo exocytosis or act on membrane-bound receptors (Dawson et al., 1992).

NO is produced upon demand within cells, along with citrulline, during the flavin and NADPH-dependent, nitric oxide synthase (NOS)-catalyzed reaction of L-arginine

with molecular oxygen (Knowles et al., 1989) (see Figure 6). Three isoforms of NOS have been identified within different cell types; neuron constitutive NOS (nNOS) (found within neurons), endothelial constitutive NOS (eNOS) (within vascular endothelial cells) and inducible NOS (iNOS) (mainly in macrophages). The activity of the two constitutive NOS forms is dependent on mobilization of intracellular Ca^{2+}, while the inducible form of NOS has a sustained Ca^{2+}-independent activity that can last for several days (Nathan and Xie, 1994). Once formed, the gaseous NO freely diffuses until it interacts with its primary target molecule, GTP. The major mechanism of NO's cell signaling action in tissue is the activation of soluble guanylate cyclase to produce cGMP from GTP(Moncada et al., 1991). The targets of cGMP action remain to be defined, however, cGMP can induce a variety of functions including protein phosphorylation, gating of cation channels, and phosphodiesterase activity (Dawson et al., 1992).

Although NO is a signaling molecule within several cell types, including enteric neurons, NO, even at physiologically normal levels, may become toxic in the presence of excessive reactive oxygen species or in a reduced antioxidant environment (Schmidt and Walter, 1994; Beckman, 1991). Nitric oxide, at the high concentrations found during inflammation (via macrophage production), is not only toxic on its own but its toxicity is enhanced by ROS or reduced antioxidant levels, as shown in Figure 6.

NO can also damage cells by inhibiting enzymes with catalytically active nonheme iron-sulfur complexes. NO inhibits both complex 1 (NAD[P]H:ubiquinone oxidoreductase) and complex 2 (succinate:ubiquinone oxidoreductase) of the mitochondrial transport complex, as well as the citric acid cycle enzyme aconitase; thus

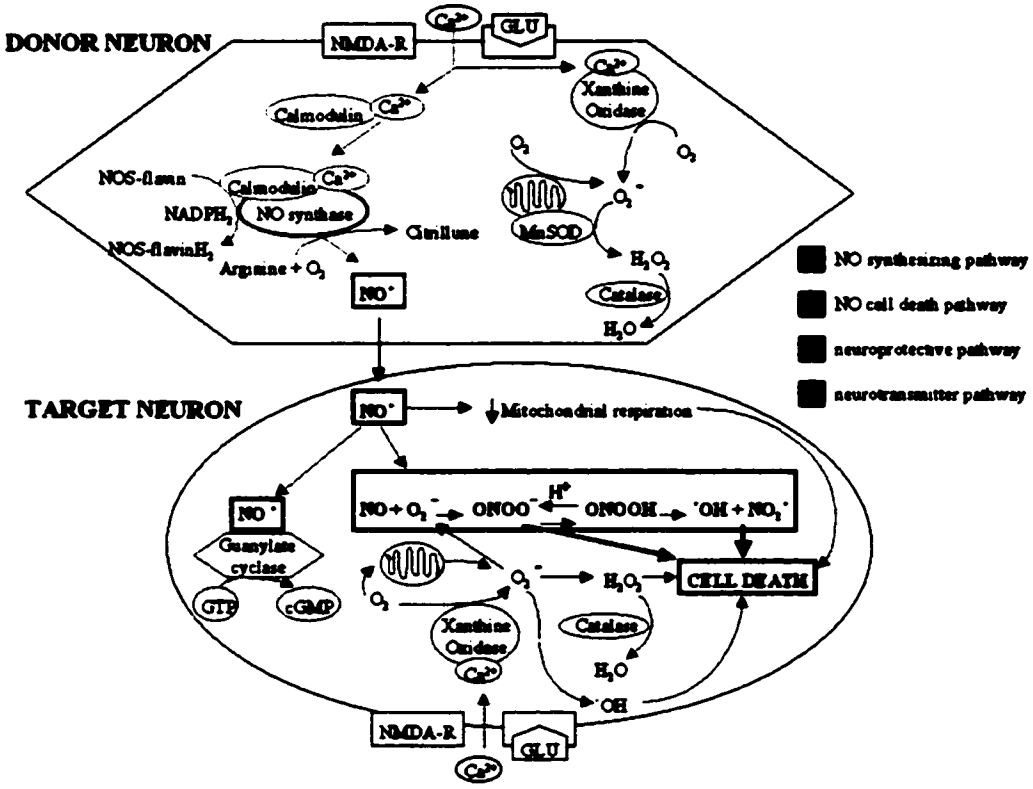

DONOR NEURON
NMDA-R
GLU
Calmodulin
Xanthine Oxidase
NOS-flavin
NADPH2
NO synthase
Citrulline
NOS-flavinH2
Arginine + O2
NO·
MnSOD
H2O2
Catalase
H2O
NO synthesizing pathway
NO cell death pathway
neuroprotective pathway
neurotransmitter pathway
TARGET NEURON
Mitochondrial respiration
Guanylate cyclase
GTP
cGMP
CELL DEATH
OH
NMDA-R
GLU

Figure 6. NO synthesis, NO-induced neurotoxicity, and neuroprotection of NOS neuron subsets. NO is synthesized along with citrulline by the reaction of arginine and O_2 in a Ca^{2+}-calmodulin, flavin and NADPH-dependent, NOS-catalyzed reaction. With sustained glutamate stimulation of NMDA receptors (NMDA-R), excessive NO is formed. NO can then freely diffuse to adjacent target neurons where it combines with the superoxide anion (O_2-) produced by mitochondria and xanthine oxidase to yield the peroxynitrite anion (ONOO-), which is an extremely potent oxidant. ONOO- is also protonated and decomposes to the hydroxyl ($OH^{\cdot}$) free radical and the nitrogen dioxide (NO_2) free radical, which are potent activators of lipid peroxidation. Although NO can function as a toxin directly, by inhibiting mitochondrial respiration via inhibition of iron-sulfur-containing enzymes, the peroxynitrite pathway may be the major pathway of cell death. NOS (donor) neurons function as "killer neurons" and possess factors that allow them to survive in a NO-rich environment. The diaphorase portion of the NOS enzyme may serve a protective role. In addition, these neurons are enriched in manganese superoxide dismutase (MnSOD), which could prevent the formation of the peroxynitrite anion, thus allowing these cells to survive (as modified from Dawson et al., 1992).

impairing mitochondrial respiration (Drapier and Hibbs, 1986; Drapier and Hibbs, 1988; Granger and Lehninger, 1982). NO also disrupts DNA synthesis by inhibition of ribonuclotide reductase, the rate-limiting enzyme in DNA replication (Nakaki et al., 1990).

In the presence of increased oxygen radical formation, NO reacts with superoxide to generate peroxynitrite, an extremely reactive molecule with potent oxidant properties and which readily diffuses across cell membranes to damage neighboring cells (Schmidt and Walter, 1994; Beckman, 1991; Beckman et al., 1990). Peroxynitrite disrupts mitochondrial electron transport (Hausladen and Fridovich, 1994), oxidizes sulfhydryl groups (Radi et al., 1991a), and initiates lipid peroxidation of enzymes and structural proteins (Radi et al., 1991b), or may degrade to hydroxyl radical, resulting in greater cytotoxicity (Beckman, 1991). NO also reacts with molecular oxygen to yield a variety of nitrogen oxides, including nitrogen dioxide and dinitrogen trioxide, which are potent oxidizing and N-nitrosylating agents. NO and NO-derived species inhibit iron-containing mitochondrial enzymes, suppress protein synthesis (Moncada et al., 1991), activate lipid peroxidation (Radi et al., 1991b), or enhance electrolyte and water secretion (Moncada and Higgs, 1993). The major source of NO that kills CNS neurons seems to derive from NOS-containing neurons, although other sources of NO have not been completely ruled out (Dawson et al., 1991; 1992).

Under normal conditions, enteric NOS activity is localized to enteric neurons and nerve fibres (Berezin et al., 1994; Kostka et al., 1993; Nichols et al., 1993; Hogaboam et al., 1995). During inflammation, granulocytes and macrophages are activated by bacterial products or by cytokines to express iNOS (Moncada et al., 1991), and are responsible for

the increased NOS expression in gut epithelia characteristic of IBD (Wong et al., 1989; Boughton-Smith et al., 1993; Grisham et al., 1994). NO has been proposed to serve an anti-inflammatory role in the normal state but to act as a mediator of injury during inflammation (Miller et al., 1993; Grisham et al., 1994). Peroxynitrite, a product of NO and ROS reaction, damages tissue and induces intestinal inflammation (Rachmilewitz et al., 1993). A definitive role for NO in ENS damage and degeneration remains to be determined. Within **Chapter 2** of this thesis, the effect of NO on the viability of enteric neurons was studied.

1.5.3 4-hydroxy-2-nonenal (HNE)

Basal levels of lipid peroxidation occur naturally as the result of metabolically derived ROS interactions with cellular membranes (Girotti, 1998). In membrane lipid peroxidation (MLP), free radicals attack the double bonds of unsaturated fatty acids that comprise membrane phospholipids (Porter et al., 1995). MLP results in the production of lipid hydroperoxides; an array of aldehydes of different carbon chain lengths. Unlike free radicals, aldehydes have long half-lives in cells and can diffuse from their site of origin to both distant subcellular sites and to other cells (Mattson, 1998). Although cellular antioxidant pathways generally detoxify these highly reactive aldehydes, overproduction of such compounds can overcome antioxidant defenses and produce significant toxicity (Keller et al., 1999).

4-hydroxy-2-nonenal (HNE) is the main aldehyde product of MLP contributing to cell death. HNE is lipid-soluble and rapidly conjugates to lysine, cysteine and histidine residues in proteins, thus disrupting normal protein function (Esterbauer et al., 1991; Waeg et al., 1996). HNE disruption of Ca^{2+}- and Na^{+}/K^{+}-ATPase and the NMDA

receptor increase intracellular Ca^{2+} levels. This in turn can induce intracellular cytotoxic processes such as oxidative stress, membrane lipid peroxidation, and mitochondrial damage (as detailed in *Section 1.4.3, Cytotoxicity*) (Mark et al., 1997a). HNE also acts directly on mitochondria, causing membrane permeabilization and triggering cellular apoptotic cascades (Kristal et al., 1996). HNE also impairs glutamate and glucose transporter activity (Keller et al., 1997; Mark et al., 1997b). Finally, by direct interaction with DNA molecules, HNE can damage DNA and inhibit DNA, RNA, and protein synthesis, glycolysis, and enzyme degradation. Thus, as depicted in Figure 7, HNE contributes to the cellular damage of MLP via multiple reaction pathways.

The role of HNE in neuronal cell death has been the subject of recent studies. Increased levels of HNE are found in several neurodegenerative diseases, including Alzheimer's and Parkinson's (Mark et al., 1997a; Girotti, 1998; Lovell et al., 1998; Markesbery and Lovell, 1998). It is cytotoxic at concentrations achieved in cells exposed to oxidative insults (Esterbauer et al., 1991), and direct application of HNE induces neuron death both *in vitro* and *in vivo* (Bruce-Keller et al., 1998; Spitz et al., 1990). Among the aldehydic products of lipid peroxidation, HNE appears to be unique in its ability to exacerbate oxidative stress, mitochondrial dysfunction, energy metabolism, and to thus contribute to oxidative stress- and MLP-induced neurodegeneration (Keller et al., 1999). This role of HNE in mediating both oxidative stress and MLP damage makes it an ideal indicator for the involvement of such mechanisms in the ENS, as performed in **Chapter 2** of this thesis.

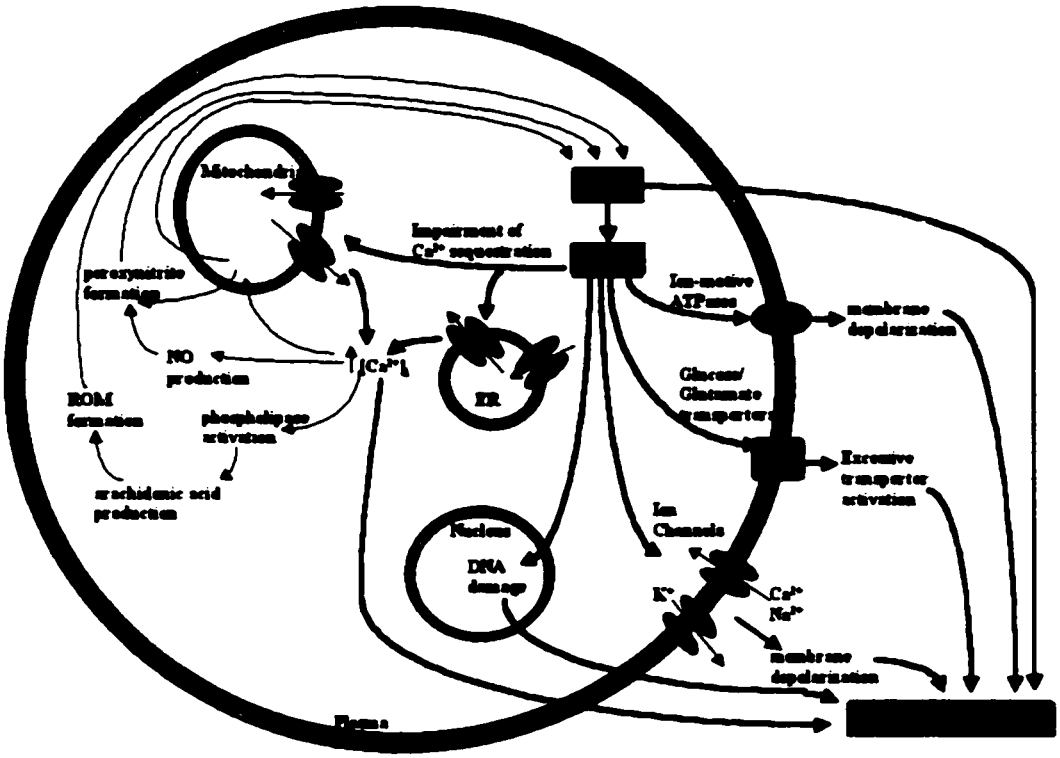

Mitochondria
Impairment of
Ca2+ sequestration
peroxynitrite
formation
NO
production
[Ca2+]i
ER
ROM
formation
phospholipase
activation
arachidonic acid
production
Nucleus
DNA
damage
Ion-motive
ATPases
membrane
depolarization
Glutamate
transporter
Excessive
transporter
activation
Ion
Channels
K+
Ca2+
Na+
membrane
depolarization
Plasma

Figure 7. Mechanisms involved in membrane lipid peroxidation (MLP) and 4-hydroxynonenal (4-HNE)-induced neurotoxicity. 4-HNE, liberated during MLP, binds to membrane transporters and ion channels, and thereby alters their activities. Alterations of the Na^+/K^+-ATPase, glucose transporter, and glutamate transporters induce membrane depolarization and excessive activation of glutamate receptors, resulting in excitotoxicity. 4-HNE also directly damages nuclear DNA and perturbs ion homeostasis in the endoplasmic reticulum (ER) and mitochondria, and thereby compromises their important Ca^{2+} sequestration functions. The resultant elevation of intracellular Ca^{2+} levels in turn promotes further MLP and 4-HNE formation by inducing nitric oxide (NO) and reactive oxygen metabolite (ROM) production.

1.5.4 Cytokines

Cytokines, which include interleukins (ILs), interferons (IFNs), growth factors and tumor necrosis factors (TNFs), are hormone-like peptides that are either released into the circulatory system to act on distant targets, or which behave as paracrine mediators that diffuse to local targets. Most cytokines are multifunctional and more than one cytokine can act on the same target cell to mediate the same or a similar function (Akira et al., 1990). These compounds are rapidly synthesized and secreted by activated leukocytes, and induce production of a variety of inflammatory mediators dependent upon the cell type (Beyaert and Fiers, 1994). Cytokine binding to specific cell-surface receptors can activate several possible intracellular second messenger systems, ultimately leading to effects on the target cell via protein kinase and/or protease pathways. Cytokines also initiate inflammation by inducing proliferation, migration, and differentiation of leukocytes, and can both prolong and/or terminate inflammation (Aggarwal and Puri, 1994). Within the immune system, these molecules modulate neuroendocrine functions and mediate interactions between immune and neuroendocrine systems (Besedovsky and Del Rey, 1992). Cytokines generally act as integral components of the immunologic defence against disease and infection. However, inappropriate cytokine expression, or a disruption in the balance between specific pro-inflammatory and anti-inflammatory cytokine expression, can induce tissue damage and disease (Tracey and Cerami, 1994).

1.5.5 Cytokines in the ENS

Study of the effects of intestinal inflammation on enteric nerve function in animal models has shown marked alterations in neurotransmitter and enzyme synthesis and

release. Marked suppression of acetylcholine production and release, suppression of norepinephrine release, and increases in immunoreactive substance P in myenteric nerves have been noted in infection-induced rat models of inflammation (Collins et al., 1989; Swain et al., 1991; 1992; Davis et al., 1998). Inducible nitric oxide synthase (iNOS) and the enzyme manganese superoxide dismutase are also rapidly induced in myenteric neurons during inflammation (Valentine et al., 1996). During inflammation, several proinflammatory cytokines including interleukin-1α, (IL-1α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF) are elaborated in the region of the myenteric plexus (Khan and Collins, 1994). Studies have thus focused on the role of these cytokines in the functional control of enteric neurons.

IL-1 has been found to have numerous effects on enteric neuronal function within a variety of experimental models. Using a longitudinal muscle-myenteric plexus preparation, IL-1β has been determined to suppress norepinephrine and acetylcholine release (Hurst and Collins, 1993; Main et al., 1993), and to increase substance P synthesis (Hurst et al., 1993). Within cultured myenteric plexus neurons, IL-1β has also been shown to induce both superoxide dismutase and iNOS expression; the coordinated induction of which is hypothesized to limit inflammatory NO cytotoxicity (Valentine et al., 1996). The actions of IL-1β on the ENS are suggested to be indirect and mediated by released prostaglandins (Bradley et al., 2000). IL-1 has been implicated as a mediator of inflammation-induced changes in myenteric nerves, as treatment of an animal model of intestinal inflammation with an IL-1 receptor antagonist attenuates inflammatory alterations in norepinephrine, acetylcholine and substance P (Jacobson et al., 1997). However, treatment with the IL-1 receptor antagonist does not fully reverse the changes

in myenteric nerve function, suggesting that other cytokines may also contribute to inflammatory alteration of myenteric nerve function.

The cytokines IL-6 and TNF are considered to be among those cytokines that contribute to inflammatory alterations of enteric nerve function. Studies have noted that IL-6 exerts a dual effect on norepinephrine release from the longitudinal muscle-myenteric plexus; at lower concentrations, IL-6 stimulates norepinephrine release, while higher concentrations inhibit norepinephrine release (Ruhl et al., 1994). Combined subthreshold concentrations of IL-1β and IL-6 also act synergistically to suppress norepinephrine release from longitudinal muscle-myenteric plexus preparations (Ruhl et al., 1994); findings consistent with the synergistic suppression of noradrenergic neurotransmission in the guinea pig submucosal plexus by these cytokines (Xia et al., 1999). IL-1β and IL-6 also suppress cholinergic neurotransmission within longitudinal muscle-myenteric plexus preparations. This action is suggested to be the electrophysiologic correlate of IL-1β and IL-6-induced suppression of ACh release (Main et al., 1993; Kelles et al., 2000). TNF, like IL-1 and IL-6, has also been shown to suppress norepinephrine release from the rat longitudinal muscle-myenteric plexus preparation. This suppression occurs via two mechanisms; early suppression involves prostaglandin actions, while late suppression is due to the synthesis and release of interleukin-1 (Hurst and Collins, 1994). Thus, the proinflammatory cytokines IL-1, IL-6 and TNF have been shown to alter neurotransmitter expression in the ENS via similar and often interacting processes, and thereby affect enteric neuronal function. Indeed, these cytokines are identified as putative mediators of altered ENS function during intestinal inflammation, and may also be involved in inflammatory degeneration of the ENS.

1.5.6 Tumor Necrosis Factor (TNF)

Tumor necrosis factor-α (TNF), also known as cachectin, is a hormone-like polypeptide with a wide range of biologic activities (Beutler and Cerami, 1987). Although this cytokine was first identified as a mediator of tumor necrosis (Carswell et al., 1975), it has since been implicated in mediating other such beneficial processes as host defence, immunity and tissue homeostasis, as well as such pathologic processes as tissue injury and chronic inflammation (Tracey et al., 1989). Thus, this pleiotropic cytokine is capable of initiating both protective and pathological actions in tissues under varying physiological conditions. Indeed, under different conditions, TNF rescues neurons from toxic insult (Cheng et al., 1994), and itself contributes to neuronal death (Perry et al., 1998; Knoblach et al., 1999). Although those factors responsible for the antithetical roles of TNF in neurodegeneration are not known, they may be related to the dual nature of inflammation: the sequestration and elimination of tissue that is too damaged to repair, and the rescue of tissues that have escaped irreparable harm (Venters et al., 2000).

TNF is widely expressed in a number of cell types including monocytes, macrophages, lymphocytes, natural killer cells, endothelial cells, mast cells, glia, astrocytes, muscle cells, fibroblasts and epithelial cells (Aggarwal, 1992; Turetskaya et al., 1992). Production of this cytokine, synthesized *de novo* following cell activation, is stimulated by endotoxins, viruses, fungal and parasitic antigens, and proinflammatory cytokines including TNF itself (Fiest et al., 1989; Beutler et al., 1986). TNF is initially synthesized as a 31 kDa precursor and proteolytic cleavage produces the secreted 17 kDa monomer, which spontaneously associates with two other such monomers to form a

biologically active trimer (Kriegler et al., 1988; Smith and Baglioni, 1987; Jones et al., 1989).

Once secreted, the biologically active TNF trimer signals target cells through binding to specific cell surface membrane receptors. TNF binding induces clustering of three receptor monomers (Banner et al., 1993). Two types of TNF receptors (TNFRs); p55 (a.k.a. TNFR1) and p75 (a.k.a. TNFR2) are present on the plasma membranes of virtually all cells. Soluble forms of both of these receptor types, known as TNF-binding proteins (TNF-BP) also exist, and their expression is associated with a variety of diseases (van Zee et al., 1992). The biological role of TNF-BPs varies; in some cases inhibiting TNF activity by competing with membrane-bound receptors for TNF, while in other cases TNF-BPs prolong and enhance TNF activity by stabilizing the trimeric structure of this cytokine (Bemelmans et al., 1993; Aderka et al., 1992).

Although the p55 and p75 TNF receptors share structural homology in their extracellular TNF-binding domains and exhibit similar binding affinities for TNF, these receptors differ in many respects. Although both receptors are present in a vast array of cell types, p55 is constitutively expressed at rather low levels, while p75 expression is inducible by a number of extracellular stimuli acting at both transcriptional and post-transcriptional levels (Vandenabeele et al., 1995). These receptors also induce separate cytoplasmic signaling following receptor-ligand binding (Smith et al., 1990; Thoma et al., 1990). Finally, these receptors differ in both their normal and pathological functional properties. While p75 can independently activate some cellular responses, including NFκB and cJun N-terminal kinases, p55 seems to be responsible for TNF signaling in most cell types (Natoli et al., 1998). For instance, under normal conditions, p55 mediates

fibroblast proliferation, antiviral activity, cellular protection, and neurotransmitter modulation (Neurath et al., 1997). Although the functions of p55 and p75 differ under normal conditions, both receptor subtypes have been implicated in the transduction of TNF-induced cell death. While TNF-induced cytotoxicity mediated through the p75 receptor has been reported (Heller et al., 1992), this receptor does not contain a cell death domain homologous to that identified in p55 (Tartaglia et al., 1993), and the primary function of this receptor is not cell death induction. In contrast, the p55 receptor has a cell death domain, induces multiple identified intracellular death pathways, and has a predominant cytotoxic function. However, the cytotoxic ability of p55 is partially dependent upon the actions of p75, with the cytotoxic actions of TNF increasing markedly in the presence of both receptor subtypes (Natoli et al., 1998). To account for observed decreases in tissue necrosis in its absence, p75 has been proposed to increase tissue sensitivity by binding TNF and presenting it to p55, which, upon TNF binding, can induce cell death through multiple death-domain-mediated pathways (Tartaglia and Goeddel, 1992).

TNF binding of p55 induces receptor aggregation, which in turn induces the formation of a multimolecular signal transducing complex from which several intracellular signaling pathways originate (Natoli et al., 1998). Separate pathways (either NFκB-dependent or -independent) can act to prevent TNF-induced cellular damage, induce gene expression, or to induce necrotic or apoptotic cell death (Natoli et al., 1998). The metabolic state of the cell influences whether transcription of protective genes or self-destruction pathways are induced (Larrick and Wright, 1990).

In all cases, TNF-binding of p55 recruits TNF-receptor 1 associated death domain containing protein (TRADD) which interacts with the death domain of p55, and acts as an adapter to recruit downstream transducers such as TNF-receptor 1 associated protein 2 (TRAF 2) (Tartaglia et al., 1993; Hsu et al., 1996). TRAF2 activates two distinct pathways that up-regulate the expression of anti-apoptotic genes and induce the synthesis of proteins that alter cell function (Natoli et al., 1998). The first such pathway involves activation of c-jun N-terminal kinase (JNK) by a mitogen-activated protein (MAP) kinase cascade (Dhanasekaran and Reddy, 1998). JNK in turn stimulates the expression of transcription factors such as c-jun and activating protein-1 (AP-1) (Luster et al., 1999). The second TRAF2-activated pathway induces nuclear factor κB-inducing kinase (NIK) activation (Malinin et al., 1997), which in turn activates the transcription factor NFκB (Hsu et al., 1996; Ting et al., 1996). Receptor-interacting protein (RIP), bound to TRADD, can also activate NFκB. These transcription factor pathways regulate the expression of genes involved in cell proliferation, inflammatory processes, and cell protection (Luster et al., 1999). These intracellular pathways, as well as the TRAF-activated inhibitor of apoptosis protein (IAP) pathway, actively prevent cell death induction by TNF (see Figure 8). Despite the lack of a death domain sequence on p75, this TNF receptor is capable of directly binding either TRAF2 homodimers or heteromeric complexes composed of TRAF1 and TRAF2 (Rothe et al., 1994; 1995). By these means, p75 can induce both JNK- and NFκB-mediated gene expression within cells (see Figure 8). Alternatively, p75 can bind TNF ligand and pass it to adjacent p55 receptors, thus increasing TNF sensitivity (Tartaglia et al., 1993; Pinckard et al., 1997).

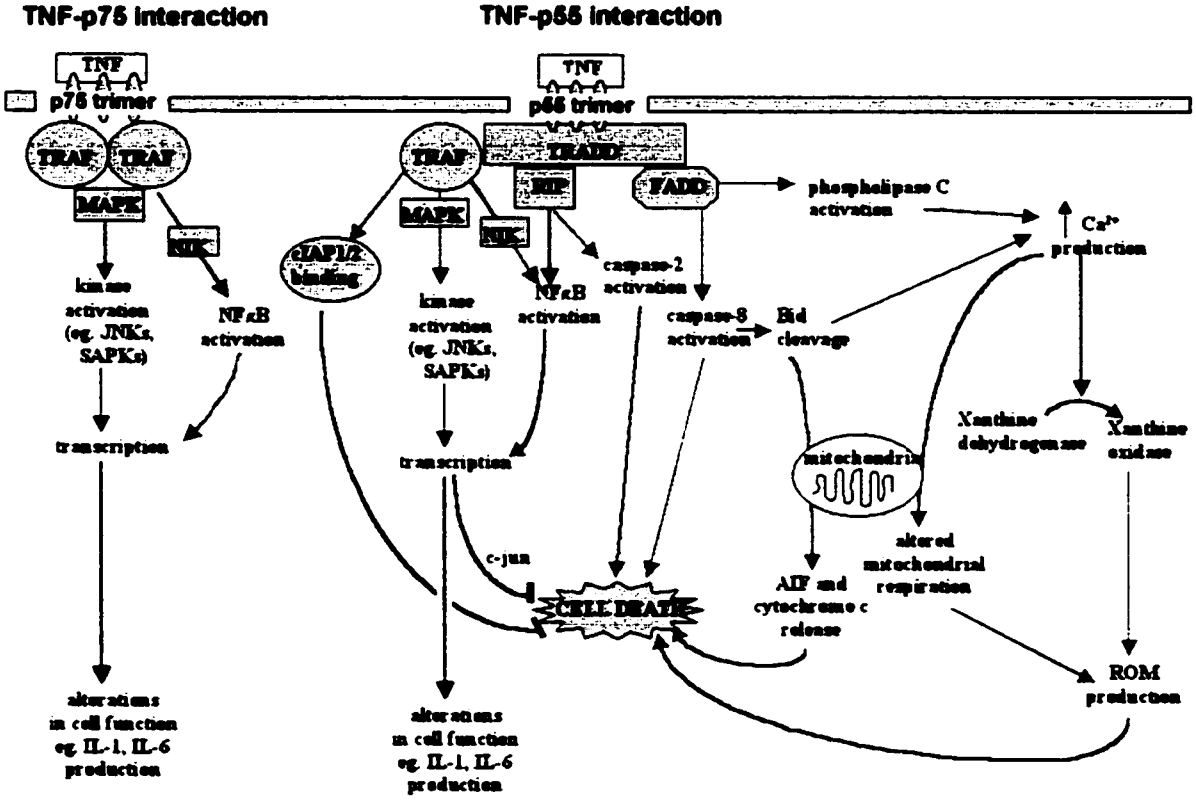

TNF-p75 interaction
TNF-p55 interaction
TNF
p75 trimer
TRAF
TRAF
MAPK
NIK
kinase activation (eg. JNKs, SAPKs)
NFκB activation
transcription
alterations in cell function eg. IL-1, IL-6 production
TNF
p55 trimer
TRAF
TRADD
RIP
FADD
cIAP1/2 binding
MAPK
NIK
kinase activation (eg. JNKs, SAPKs)
NFκB activation
caspase-2 activation
caspase-8 activation
Bid cleavage
transcription
alterations in cell function eg. IL-1, IL-6 production
c-jun
CELL DEATH
phospholipase C activation
Ca2+ production
mitochondria
AIF and cytochrome c release
altered mitochondrial respiration
Xanthine dehydrogenase
Xanthine oxidase
ROM production

Figure 8. Cytotoxic, non-cytotoxic and anti-cytotoxic intracellular mechanisms of TNF actions. TNF trimers can directly bind with either trimeric p55 or trimeric p75 receptor subtypes to induce a variety of intracellular actions. TNF binding of trimeric p75 can induce alterations in cell function via either TRAF-MAPK kinase activation or via NIK-induced NFκB activation pathways. TNF binding of trimeric p55, which is bound to the TRADD adaptor, recruits the signal transducers FADD and TRAF. FADD can activate a phospholipase C-cytotoxic pathway that induces cell death through mitochondrial damage and oxidative stress. FADD can also interact with the apoptotic protease caspase-8 to induce apoptosis directly. Alternatively, caspase-8 activation may also act to cleave Bid, a proapoptotic protein. The resulting c-terminal fragment translocates to the mitochondria, releasing cytochrome c and apoptosis-inducing factor (AIF), which in turn induce cell death. Finally, cell death can be induced via receptor-interacting protein (RIP) activation of caspase-2 apoptotic cascades. TNF activation of gene transcription may both alter cellular function and inhibit cell death pathways. These intracellular pathways of gene transcription involve both RIP and the signal transducer TRAF. RIP activates the transcription factor NFκB, inducing transcription of a large number of possible genes, dependent on the cell type. These genes may act to prevent cell death, or to alter cell function. TRAF, upon binding the apoptosis-inhibiting protein cIAP1/2, can inhibit apoptosis. TRAF can also bind the NFκB-inducing kinase NIK, inducing kinase activation and gene transcription. Such diversity of intracellular mechanisms of action underlies the variety of TNF actions within cells.

Depending on the target cell, TNF can induce either necrotic or apoptotic cell death (Laster et al., 1988). Apoptotic cascades can be directly induced by TRADD binding of the downstream transducer Fas-associated death domain protein (FADD). FADD directly interacts with apoptotic proteases to trigger cell death via a number of possible apoptotic and necrotic pathways (as detailed in *Section 1.7, Cell Death Mechanisms*) (Muzio et al., 1996; Boldin et al., 1996). TRADD binding of RIP also induces caspase-2 mediated apoptotic pathways (Scheurich et al., 1989; Luster et al., 1999) (see Figure 8).

Due to its multiple intracellular signaling pathways, TNF is able to exert pleiotropic effects on a large number of different cell types (Camussi et al., 1991). These effects may be either injurious or beneficial, dependent upon the relative concentration of TNF, duration of exposure, presence of other mediators that may affect the activity of TNF, and the cell type involved (Ledgerwood et al., 1999; Tracey et al., 1989). The major physiological functions of TNF are not cytotoxicity, but rather synthesis of gene products that influence cellular differentiation, growth stimulation, antiviral activity, immunomodulation, and inflammation (Beyaert and Fiers, 1998; Zhang and Tracey, 1998). The cytotoxic actions of TNF underlie the detrimental actions of cachexia, septic shock, endotoxin-induced tissue injury, and the damage associated with chronic inflammation (Tracey et al., 1988; 1989). Such antithetical roles of TNF in both protecting tissues and destroying them may be related to the dual nature of inflammation: rescuing tissues that have escaped irreparable harm, and sequestering and eliminating tissue that is too damaged to repair (Venters et al., 2000).

Because of the role that chronic inflammation plays in many diseases, the function of TNF in regulating inflammatory processes has been extensively investigated (Vassali, 1992; Bazzoni and Beutler, 1996; Aggarwal and Natarajan, 1996). TNF is a pivotal mediator of inflammation that activates leukocytes, enhances adherence of neutrophils and monocytes to endothelium, promotes migration of inflammatory cells into the intercellular matrix, stimulates fibroblast proliferation, and triggers the local production of other proinflammatory cytokines (Tracey and Cerami, 1994). These TNF actions are thought to contribute to infection containment, destruction of malignant cells, and tissue regeneration during inflammatory processes. However, overproduction of TNF, as during chronic inflammation, manifests an inflammatory component that exacerbates disease severity (Beyaert and Fiers, 1998; Zhang and Tracey, 1998).

Within inflammatory bowel disease (IBD), TNF acts as a pivotal inflammatory mediator central to disease induction and maintenance (Stokkers et al., 1996). Indeed, anti-TNF antibody therapy is currently in use in clinical trials for IBD (Van Dullemen et al., 1995; Murthy et al., 1999). The pathologic role of TNF within inflammatory damage of the gastrointestinal tract makes this cytokine a likely candidate to act as a neurodegenerative agent in the gastrointestinal ENS. While no such investigations into TNF's neurodegenerative properties in the ENS have yet been performed, TNF does possess neurodegenerative properties in the diseased CNS. In the CNS, TNF initiates inflammatory cascades leading to neuronal apoptosis and neurological impairment (Perry et al., 1998; Knoblach et al., 1999). Given its known neurodegenerative properties and its cytotoxic role in chronic bowel inflammation, a role for TNF in mediating

inflammatory damage to the ENS is highly likely and represents the focus of my research described in **Chapter 3.**

1.6 Neuroprotection

1.6.1 Neurotrophic Factors

Neurotrophic factors, also called neurotrophins, are endogenous soluble polypeptides that regulate long-term survival and differentiation of neurons of the peripheral and central nervous systems (Shen et al., 1997). These polypeptides are signaling molecules that interact with specific cellular receptors leading to biological responses that include cellular proliferation, cell differentiation, and changes in cell motility, cell structure, or general cellular phenotype (Semkova and Krieglstein, 1999). Neurotrophic factors specifically influence neuronal activity by promoting neuron development and maturation during embryonic life, and by sustaining these cells during adult life and promoting neurite regeneration after injury (Terenghi, 1999). However, as reflected in the changing expression patterns of neurotrophic factors, some neuronal populations are sustained during development by a given trophic factor and then switch to a different trophic for support in adult life (Maisonpierre et al., 1990; Zhang et al., 1994; Oakley et al., 1995; Verdi et al., 1996). Neurotrophic factors can also protect and even restore impaired cellular functions resulting from trauma, aging, toxic agents, and genetically linked neurodegenerative disorders (Shen et al., 1997). As such, they have been advocated as ideally suited for the treatment of neurodegenerative diseases of the CNS, neuropathies and peripheral nerve injury (Anand, 1996; Lindsay, 1996).

Neurotrophic factors are subdivided into families according to their sequence similarity and to the type of binding receptors with which they interact (Hopkins and

Rothwell, 1995). By these means, several families of neurotrophic factors have been identified. The first family is the neurotrophins; structurally related secretory proteins that are widely expressed in neurons and their target cells (Korsching, 1993; Gotz et al., 1994). To date, five neurotrophin family members have been identified; nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophins (NT) 3, 4/5, and 6. These are signaling molecules that share a high degree of amino acid homology, and function by binding to their respective cell surface tyrosine kinase receptors. The second family of neurotrophic factors is the ciliary neurotrophic factor (CNTF) family that include CNTF, leukemia inhibitory factor (LIF), interleukins 6 and 11, and cardiotropin 1. These neurotrophic factors interact with multi-component receptors possessing a common subunit for all members of the CNTF family, as well as other subunits unique for their respective factors (Stahl and Yancopoulos, 1994; Shen et al., 1997). The third identified neurotrophic factor family is the glial cell-line derived neurotrophic factor (GDNF) family. There are four known members of the GDNF protein family: GDNF, neurturin (NTN), persephin (PSP), and artemin (ARTN). All four are potent survival factors for cultured peripheral and/or central nervous system neurons (Rosenthal, 1999).

1.6.2 Neurotrophic Factors in the ENS

While a large number of neurotrophic factors have been identified within vertebrates, localization of such factors to the enteric nervous system and identification of their function in the ENS is ongoing. Neurotrophin-3 (NT-3), the most abundant neurotrophin during early development (Farinas et al., 1994), signals a number of trophic effects through its transducing receptor tyrosine kinase TrkC (Chalazonitis, 1996).

Within the developing ENS, NT-3 promotes differentiation of neural crest-derived precursors cells into neurons and glia (Chalazonitis et al., 1994; 1998). As more enteric nerve cells are produced in the presence of NT-3, but NT-3 does not induce precursor proliferation, NT-3 must increase the proportion of precursors that differentiate into neurons or glia (Gershon, 1998). Although mice lacking NT-3 have severe deficits in sensory and sympathetic neuronal populations, the enteric nervous system develops normally; indicating that NT-3 is not essential to ENS development (Farinas et al., 1994). Either compensatory mechanisms can substitute for the loss of NT-3 during development, or NT-3 is involved in the development of only a relatively small subset of enteric neurons or glia (Gershon, 1998). Postnatally, both NT-3 and its TrkC receptor are expressed in the rat gut at a time when changes in the density of intestinal innervation are occurring. NT-3 treatment promotes neuronal survival and neurite outgrowth, suggesting that NT-3 may play a role in postnatal ENS development (Saffrey et al., 2000). Studies localizing NT-3 to specific neuronal subtypes (VIP-positive, Substance P/tachykinin-negative), and the trkC receptor to VIP or Substance P/tachykinin-positive neurons also suggest that NT-3 and its receptor may be involved in the maintenance of mature enteric neural circuits, transmission, and phenotypic expression (De Giorgio et al., 2000). NT-3, along with brain-derived neurotrophic factor (BDNF) and their receptors TrkA, TrkB, and TrkC, have been localized to the human ENS from infancy to adulthood (Hoehner et al., 1996a). Hirschsprung's disease is characterized by the congenital absence of enteric neurons. The marked downregulation of both these neurotrophic factors and their receptors in the ENS of Hirschsprung's disease patients (Hoechner et al.,

1996b) suggests that NT-3 and/or BDNF may be of potential importance in functional differentiation disorders of the intestinal ENS.

Recent investigations have focused on identification of the role of the GDNF homologue neurturin in the enteric nervous system. Both GDNF and neurturin support the survival and proliferation of both glial and enteric precursors *in vitro* (Heuckeroth et al., 1998; Taraviras et al., 1999); indicating that these neurotrophic factors may be important for ENS development. Findings in both neurturin-receptor-deficient and neurturin-deficient mice support this hypothesis, as these mice have reduced myenteric plexus innervation density and reduced gastrointestinal motility (Rossi et al., 1999; Heuckeroth et al., 1999). Clinical findings have implicated mutations in the neurturin gene, in conjunction with gene mutations in its signaling component Ret, in the pathogenesis of Hirschsprung's disease (Doray et al., 1998). A functional role may also exist for neurturin in the adult ENS, as neurturin mRNA and protein are expressed within the adult murine intestine (Xian et al., 1999; Golden et al., 1999).

Finally, some sparse evidence suggests that additional neurotrophic factors may play important roles in the enteric nervous system. Ciliary neurotrophic factor (CTNF) and leukemia inhibitory factor (LIF) promote the development of ENS neurons *in vitro*; suggesting that these neurotrophic factors may also be involved in *in vivo* ENS development (Chalazonitis et al., 1998). Nerve growth factor (NGF), which has important developmental actions in both the central and peripheral nervous systems, also has suggested roles in the differentiation and development of the postnatal ENS. NGF addition to cultured postnatal enteric neurons stimulates neurite outgrowth (Mulholland et al., 1994), and its removal from the postnatal ileum alters neurotransmitter expression

patterns within the ENS (Belai et al., 1992). Additionally, Trk proteins, essential constituents of high-affinity neurotrophin receptors, and p75 protein, a low affinity neurotrophin receptor, have been localized to neuronal and glial populations within the gut of adult mammals (Esteban et al., 1998). Thus, the actions of specific neurotrophins on their respective receptors may be involved in the physiology and/or pathology of the adult ENS.

1.6.3 Glial Cell-line Derived Neurotrophic Factor (GDNF)

The neurotrophic factor GDNF was first identified due to its protective and restorative effects on mesencephalic dopaminergic neurons in culture (Tomac et al., 1995). Additional neuronal targets have since been identified, including motor neurons, noradrenergic neurons, cholinergic neurons, serotonergic neurons, sympathetic and ciliary neurons, sensory neurons, and enteric neurons (Henderson et al., 1994; Arenas et al., 1995; Pichel et al., 1996). The requirement for GDNF in the normal development of these neurons (other than dopaminergic neurons) has been demonstrated in GDNF knockout mice (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996), while studies indicate the neuroprotective role of this factor in the adult (Oppenheim et al., 1995).

GDNF is essential in the development of the fetal ENS. This neurotrophic factor is critical for migration of vagal neural crest cells into the hindgut and their maturation to form the enteric innervation of this region (Shen et al., 1997; Schuchardt et al., 1994). Like NT-3, GDNF promotes the development and survival of enteric neurons, but unlike NT-3, GDNF also causes the neural crest-derived precursors of these cells to proliferate. Accordingly, mutations of either the GDNF gene (Pichel et al., 1996; Senchez et al.,

1996) or the gene for its receptor c-ret (Schuchardt et al., 1994) prevent ENS development below the level of the rostral foregut. In humans, mutations of these genes give rise to Hirschsprung's disease, which is characterized by absence of the intrinsic ENS innervation in the gut (Martucciello et al., 1998 Pasini et al., 1996). Early in development, GDNF acts as a mitogen (Chalazonitis et al., 1997), greatly expanding the numbers of enteric crest-derived neural precursors, and may also promote the migration of these crest cell throughout the gastrointestinal tract (Young et al., 2001). Later during development, GDNF loses its ability to promote proliferation and acts only as a growth-differentiation factor, supporting enteric neuronal but not glial development. Thus, of the known neurotrophic factors in the ENS, GDNF is the only factor that is essential for the development of all enteric neurons and glia.

Although GDNF's role as a neurotrophic factor has been identified, the mechanisms by which GDNF functions, including its interaction(s) with receptor(s) and the pathways involved in its neuroprotective actions, are incompletely characterized. The family of GDNF ligands (GDNF, NTN, ART and PSP), are thought to mediate their activities through a family of multicomponent receptors that are composed of the transmembrane tyrosine kinase Ret and one of four glycosyl-phosphatidyl inositol (GPI) linked proteins, designated GFR α1 to GFR α4 (a.k.a. GDNFR1 to GDNFR4 or RETL1 to RETL4) (Sanicola et al., 1997). According to this hypothesis Ret, which is expressed in all cell populations that respond to GDNF (Pachnis et al., 1993), is shared by all the GDNF ligand receptors and functions as a signaling component, whereas the GFRαs are receptor-specific and act as ligand binding components. Although Ret alone does not bind any of the GDNF ligands, it is capable of modifying the interactions between these

ligands and the GFRαs. Thus, in the absence of Ret, each GFRα binds only a single ligand, whereas in its presence they interact with multiple GDNF ligands (Rosenthal, 1999). Although GDNF itself can interact with multiple GFRα complexes *in vitro*, it interacts almost exclusively with GFRα1 complexes *in vivo* (Rossi et al., 1999). Once GDNF has bound GFRα1, it is thought to recruit c-ret into the ligand-receptor complex, and induce c-ret tyrosine phosphorylation (Treanor et al., 1996; Jing et al., 1996). Following c-ret activation, the subsequent intracellular mechanisms that ultimately result in the neuroprotective ability of GDNF remain to be conclusively identified.

Experiments have shown GDNF to protect against apoptotic, but not necrotic cell death. The anti-apoptotic effects of GDNF are thought to involve suppression of oxygen radical accumulation, which is the apoptotic process upstream of caspase-3 activation. GDNF-receptor interactions are thought to activate phosphatidylinositol 3-kinase, upregulating Bcl-2 and Bcl-x which in turn suppress the intracellular accumulation of ROS and thus the subsequent induction of caspase-3 apoptotic pathways (Sawada et al., 2000). However, GDNF is selective in its protective abilities; preventing induction of caspase-3 apoptotic pathways, but demonstrating no neuroprotective effect against insults that directly activate apoptotic cascades downstream of caspase-3 activation (Sawada et al., 2000). Thus, much remains to be learned about GDNF and its neuroprotective properties.

Our particular interest in GDNF stems from its unresolved function in the adult gut and enteric nervous system (ENS). Although overall GDNF levels decrease during development, they increase specifically within the gastrointestinal tract and remain high within adult GI tissue (Peters et al., 1998). Much of this gastrointestinal GDNF

production has been localized to gastrointestinal smooth muscle (Peters et al., 1998). Within the gastrointestinal tract, the GDNF receptor Ret is localized to enteric neurons, but not associated glia; identifying these neurons as likely targets of GDNF actions (Bar et al., 1997). While the role of GDNF in the adult gut is presently unknown, I hypothesize that, similar to its neuroprotective role in adult CNS and other autonomic neurons, GDNF is involved in the maintenance and neuroprotection of the adult ENS (Beck et al., 1995; Bowenkamp et al., 1995; Tomac et al., 1995; Li et al., 1995; Clarkson et al., 1995; 1997). A study of the neuroprotective ability of GDNF within the ENS is detailed in **Chapter 2** of this thesis.

1.7 Cell Death Mechanisms

Cell death occurs via two known mechanisms; apoptosis and necrosis. Necrosis is a passive, catabolic process that represents cellular response to pathological injury or toxic insult (Renvoise et al., 1998). A number of damage pathways have been implicated in necrosis, including generation of toxic levels of reactive oxygen species, phospholipase activation, perturbation of Ca^{2+} homeostasis, ATP depletion, and unspecific DNA and protein damage (as reviewed in Beyaert and Fiers, 1994; Wallach et al., 1997). In contrast to the passive nature of necrosis, apoptosis is an active process, effected by the cell itself and requiring energy to do so. Apoptosis can occur via physiological means, as during embryogenesis, metamorphosis, and normal cell turnover, in a process also known as programmed cell death (Toescu, 1998; Renvoize et al., 1998). However, apoptosis can also be elicited pathologically by toxic injury or during disease pathogenesis (Thompson, 1995).

The morphological changes that occur during apoptosis are distinctly different from those of necrosis, and are the central standard for both identifying this cell death mechanism and for distinguishing it from necrosis (Renvoize et al., 1998). One of the earliest events in apoptosis is cell shrinkage concomitant with loss of contact with adjacent cells. Nuclear chromatin then condenses against the nuclear membrane, followed by nuclear fragmentation into compact chromatin "beads" scattered throughout the cytoplasm. Next, the plasma membrane of the cell begins to convolute and to form apoptotic bodies, (cellular processes containing condensed nuclear fragments and cytoplasmic constituents all surrounded by plasma membrane) which are pinched off and separate from the cell in a process known as budding. Finally, dying cells and apoptotic bodies are rapidly engulfed by neighboring cells without eliciting any inflammatory reaction (Renvoize et al., 1998).

Unlike apoptosis, cellular homeostasis is lost early in necrosis, and is followed by swelling of the entire cell and its organelles, then by plasma membrane rupture, mitochondrial dysfunction, release of intracellular constituents including proteolytic enzymes, and the slow dissolution of the nucleus (Kerr et al., 1972). This cellular degradation commonly induces an inflammatory response, with activated immune cells phagocytosing cellular remains.

Based on their morphological and metabolic differences, apoptosis and necrosis have traditionally been viewed as two distinct types of cellular responses to injury, regulated by different intracellular mechanisms, with no degree of overlap (Buja et al., 1993). However, both these processes can occur simultaneously in tissues exposed to a given stimulus, with the intensity of the pathological insult and the energy supply of each

individual cell determining whether that cell undergoes apoptosis or necrosis (Lennon et al., 1991; Bonfoco et al., 1995; Shimizu et al., 1996; Leist et al., 1997; Eguchi et al., 1997). It has been proposed that at least some of the early intracellular damage events are common to both types of cell death, and that downstream controllers may decide which cell death mechanism prevails. Thus, apoptosis and necrosis may either represent two essentially separate processes with a small degree of overlap, or may be two extremes of a continuum of cellular death (Richter et al., 1996; Leist and Nicotera, 1997; Tsujimoto, 1997).

Cytotoxic insults common to both necrosis and apoptosis include the actions of excitatory amino acids (as detailed in section *1.4.3 Cytotoxicity*). Overactivation of excitatory amino acid receptors, notably NMDA receptors, stimulates excess Ca^{2+} influx into cells. Elevation of intracellular Ca^{2+} levels represents a relatively common trigger for apoptosis in cells of diverse tissue origins (Nicotera et al., 1994; 1996). Ca^{2+} can trigger apoptotic caspase cascades, or activate apoptotic mitogen-activated protein kinase (MAPK) signaling pathways (Lipton, 1997; Kawasaki et al., 1997). These caspase and kinase apoptotic pathways interact in cases where caspases are required for kinase activation, which in turn increases caspase activity, comprising a positive feedback loop (Cardone et al., 1997). Elevated Ca^{2+} levels can also induce further intracellular damage, such as oxygen free radical and NO generation, mitochondrial damage, and nuclear alterations, which can induce either necrosis or apoptosis (Bonfoco et al., 1995; Ankarcrona et al., 1995).

Oxidative stress, which can be induced by multiple pathways, can itself induce either apoptosis or necrosis (Dypbukt et al., 1994). When levels of highly reactive oxygen

radicals, such as superoxide and hydrogen peroxide are increased, these molecules can induce cell death via several pathways including caspase activation, DNA binding of transcription factors, and cytoskeletal alterations (Abate et al., 1990; Meyer et al., 1993; Troy et al., 1996). Similarly, elevated levels of NO can react with superoxide to form the highly cytotoxic peroxynitrite radical, which can also induce either apoptosis or necrosis by similar mechanisms (Bonfoco et al., 1995).

Apoptosis can be selectively and specifically induced within cells by ligand interaction with cell surface receptors specialized to induce cytotoxic signals. Such receptors commonly possess a wide range of other functions unrelated to cell death, with apoptosis induced only in specific cell types under highly regulated conditions (Schulz-Osthoff et al., 1998). These receptors are capable of inducing multiple apoptosis-inducing pathways within a given cell. For example, some members of the tumor necrosis factor (TNF) receptor superfamily have known apoptosis-inducing activities. These receptors include APO-1/Fas (a.k.a. CD95), TNF-receptor 1 (TNF-R1), TNF-related apoptosis-inducing ligand–receptor 1 (TRAIL-R1), TNF-related apoptosis-inducing ligand–receptor 2 (TRAIL-R2), and TNF-receptor-related apoptosis-mediating protein (TRAMP) (Itoh et al., 1991; Oehm et al., 1992; Kitson et al., 1996; Pan et al., 1997). These receptors all contain a similar amino acid sequence known as the death domain that enables transmission of cytotoxic signals (Tartaglia et al., 1993; Itoh and Nagata, 1993). Following binding of their respective ligands, the receptor death domains recruit other intracellular death domain-containing proteins which then serve as adapters for apoptotic signal cascades (Schulz-Osthoff et al., 1998). Known death domain-containing adapter proteins involved in apoptosis include Fas-associated death domain

protein (FADD) (a.k.a. MORT1), receptor-interacting protein (RIP), RIP-associated ICH-1/Ced-3 homologous death domain protein (RAIDD) (a.k.a. CRADD), and TNF receptor adapter death domain (TRADD), which are all activated by most of the known death-domain containing receptors (Chinnaiyan et al., 1995; Stanger et al., 1995; Hsu et al., 1995; Duan and Dixit, 1997; Ahmad and Goldstein, 1997). These adapter proteins commonly activate apoptotic caspase cascades, either directly or through protein kinase-mediated pathways (Muzio et al., 1996).

The caspase family of cysteine proteases is considered to act as key effector molecules in the majority of apoptotic pathways (Schulz-Osthoff et al., 1998). These molecules, which are related to the nematode gene *ced-3* and the human interleukin-1β converting enzyme (ICE), are synthesized as inactive precursors that need to be activated by proteolytic cleavage (Yuan et al., 1993). Caspase cascades can act either as initiators or executioners of apoptosis. Apoptotic initiators, such as caspase-8 and caspase-10, act upstream of mitochondrial processes in apoptotic pathways. These caspases are activated through either death-receptor pathways or by excitotoxic intracellular messengers such as Ca^{2+}, cAMP, and free radicals including nitric oxide and superoxide (Lipton and Nicotera, 1998). Once activated, initiator caspase cascades induce pro-apoptotic members of the Bcl-2 protein family to open the mitochondrial permeability transition (Li et al., 1998; Susin et al., 1998). Specific caspases that act as executioners of apoptosis, such as caspase-3 and caspase-9, act downstream of mitochondrial permeability transition opening. These caspases are activated by released mitochondrial factors to initiate the final execution stage of apoptosis. Within this final stage of apoptosis, cleavage of the DNA repair enzyme poly (ADP-ribose) polymerase (PARP), internucleosomal DNA

hydrolysis, cell shrinkage, chromatin margination, and nuclear lobulation all commonly occur (Lemasters et al., 1999).

Mitochondrial alterations play a critical role in both necrosis and apoptosis, as rapid and dramatic decreases in mitochondrial potential are observed in both forms of cell death (Kroemer, 1997; Hirsch et al., 1997). Mitochondrial permeability transition pore (PTP) opening, which permits molecules to be released from the mitochondrial matrix, underlies this drop in mitochondrial potential. PTP opening can be induced by several means. Nuclear alterations, such as DNA damage, can activate the mitosis inhibitor protein p53 to induce PTP opening via the actions of reactive oxygen species or ceramide (Polyak et al., 1997). Alternatively, "initiator" caspase cascades, activated by several possible means, can directly induce PTP opening.

Upon PTP opening, apoptogenic factors are released from the mitochondrial intermembrane space and leak into the cytosol. Release of apoptosis-inducing factor (AIF) and Cytochrome c (which binds apoptosis-activating factor-1 and ATP) activates "executioner caspases" including caspase-9 and caspase-3 which initiate DNA degradation and nuclear apoptosis (Susin et al., 1998; 1999). PTP opening also causes major changes in cellular redox potentials, depletion of energy sources such as ATP, and dramatic changes in intracellular ion concentrations. During necrotic cell death, PTP opening may lead to increased radical production, which in turn causes cell damage via the oxidation of lipids, proteins and DNA (Schulz-Osthoff et al., 1998). Although it is not entirely clear which event decides whether a cell undergoes apoptosis or necrosis, the supply of ATP and other energy equivalents are likely determinants (Leist et al., 1997). Necrosis preferentially occurs when rapid onset of PTP opening causes ATP depletion,

whereas apoptosis develops when PTP opening occurs without exhaustion of ATP (Lemasters et al., 1999). Whether apoptosis or necrosis ensues, PTP opening and the subsequent cellular damage rapidly induce the characteristic morphological changes of the specific cell death mechanism.

It is important to discriminate between apoptosis and necrosis in pathological conditions and to assess the relative contribution of each of these cell death mechanisms to overall tissue damage. Such information contributes to the knowledge and characterization of toxicological insults and disease pathogenesis, and may be relevant when designing therapeutic treatments specifically targeting either apoptotic or necrotic injury (Nicholson, 1996; Renvoize et al., 1998). In my investigation of the neuropathologic properties of specific mediators of inflammation within the ENS, I have also undertaken a study of potential cell death mechanisms (described in **Chapters 2 & 3** of this thesis).

1.8 Summary

Knowledge of enteric neuronal degeneration and the resultant impairment of gastrointestinal function within disease pathogenesis currently far outweighs any insight into the mechanisms underlying such neurodegeneration. Identification of neurotoxicological agent(s) involved in clinically observed gut neuropathogenesis represents a necessary initial step towards the development of effective therapies that prevent or attenuate ENS damage. Currently, no such therapies are available, despite the long-lasting and frequently permanent nature of gastrointestinal symptoms induced by ENS damage.

Recently, clinically observed associations between intestinal inflammatory processes and ENS damage have been substantiated by the experimental determination of a causal relationship between inflammation and enteric neurodegeneration in experimental models. However, at the present time there is a paucity of data on the role that specific inflammatory mediators play in ENS neurodegenerative processes.

The multitude of compounds involved in inflammatory processes realistically prohibits an all-inclusive examination of the neurotoxicological properties of all identified inflammatory mediators. However, some comprehensive examination is required to identify compounds with clinically relevant toxicities in the ENS. Identification of such compounds would provide a foundation upon which rational therapies for the treatment of enteric damage and degeneration could be developed.

The initial challenge of this study was the identification of candidate molecules that could induce degeneration of enteric neurons. The criteria by which molecules were selected for study were 1) an identified role in the induction of neurodegeneration within the CNS, to which the ENS bears structural and functional resemblance, 2) a primary role in the pathogenesis of inflammatory tissue damage within intestinal disease, and, if possible, 3) some evidence suggesting involvement in ENS pathogenesis. Based upon these criteria, reactive oxygen species including hydrogen peroxide (H_2O_2) and nitric oxide (NO), the cytokine tumor necrosis factor (TNF), as well as the processes of oxidative stress and hypoxia-reoxygenation were selected for investigation of their neurotoxic actions within the ENS. Characterization of *in vitro* neurotoxicity included identification of cell-type specificity of action(s), establishment of toxicity concentration

curves, identification of cell death mechanisms, and investigation of the neuroprotective ability of the putative enteric neuroprotectant GDNF.

1.9 Aims of this thesis

Inflammation is a highly complex process involving several interacting processes and alterations in the secretion of a variety of mediators. Thus a multitude of compounds secreted during gut inflammation may be involved in affecting enteric neuron function and viability. The general aim of this thesis was to identify specific inflammatory mediators that induce enteric neurodegeneration, and to characterize their neurodegenerative properties. Such findings are intended to contribute towards the ultimate goal of this research; the development of a specific therapeutic treatment to prevent or attenuate ENS damage in gut inflammatory disease. To that end, the research objectives of this thesis were threefold. The first objective was the development of a suitable *in vitro* system for the qualitative and quantitative study of neurodegeneration within the adult mammalian ENS, as performed in **Chapter 2** of this thesis. The *in vitro* assay developed was based on the primary culture of adult enteric neurons, and this was further facilitated by development of a process for enteric nerve cell cryopreservation storage. The second objective was elucidation of the neurodegenerative actions in the ENS of reactive oxygen species (**Chapter 2**) and the process of hypoxia-reoxygenation (**Chapter 2**). Specific characteristics of such experimentally induced neurodegeneration including cell death mechanisms and the ability of neurotrophic factors to protect these neurons against damage were investigated. Finally, the proinflammatory cytokine tumor necrosis factor (TNF), a molecule of primary importance in IBD pathogenesis, was studied extensively to determine its role in enteric neurodegeneration (**Chapter 3**).

These studies included examination of macrophage involvement in such processes. In addition, TNF sensitization phenomena were investigated for possible relevance to the mechanisms underlying intestinal disease pathogenesis.

Chapter 2

Optimization Of Primary Myenteric Neuronal Cell Cultures From Adult Rat Ileum For Neurotoxicological Experimentation, And Examination Of The Role Of Specific Inflammatory Mediators And Neurotrophic Factors In ENS Neurodegenerative Processes Using This Cell Culture System.

Please note that the cryopreservation protocol for this chapter is being withheld by the university for proprietary considerations. For this reason, any discussion pertaining to the cryopreservation procedure has been removed from this thesis.

Summary

In order to permit the study of neurotoxicity and neuroprotection in the isolated adult enteric nervous system, we have developed a procedure for culturing dissociated enteric neurons from the myenteric plexus of adult rat ileum specifically optimized for use in neurotoxicological experiments. These monolayer cultures consist of adherent neuron-glial cell aggregates that resemble enteric ganglia *in vivo* by morphological, electrophysiological, and neurochemical criteria. These cultures permit quantitative evaluation and characterization of injury, without the problem of substrate degradation upon insult commonly observed in cultures.

To facilitate the use of such adult ENS cultures, a protocol has also been developed for the cryopreservation and storage of dissociated neurons from the myenteric plexus of adult rat ileum. The viability of both enteric neuron and non-neuronal cell populations within these cryopreserved cultures does not significantly differ from those of cell populations within freshly plated control cultures. Cryopreserved neurons exhibit those morphological, electrophysiological, and neurochemical characteristics of freshly plated cultures. This cryopreservation procedure should contribute substantially to the efficacy of study of the adult enteric nervous system *in vitro*.

The use of isolated cultures of the adult ENS for neurotoxicological experimentation represents an extremely useful system for elucidation of the mechanisms underlying clinical ENS degeneration. Such damage and degeneration of the adult ENS, which occurs via currently unknown mechanisms, is a hallmark of several diseases of the adult gastrointestinal tract including inflammatory bowel disease and hypoxia/ischemia, as well as several extra-intestinal diseases. Use of adult ENS cultures also readily

permits elucidation of neurotrophic factors that may prevent or attenuate ENS damage in such diseases.

In this study, we have attempted to elucidate possible mechanisms involved in ENS toxicity. Oxidative stress and lipid peroxidation processes are involved in the pathogenesis of inflammatory bowel disease (IBD), in which enteric neuronal degeneration commonly occurs. We hypothesized that such inflammatory processes may also induce ENS degeneration. The neurotoxicity of 4-hydroxynonenal (HNE), hydrogen peroxide (H_2O_2), menadione, and sodium nitroprusside (SNP) were thus evaluated in primary cultures of rat myenteric neurons. Total and neuronal cell loss, apoptosis and necrosis were evaluated by histochemical and immunohistochemical labeling. Each of the toxins tested induced extensive cell loss and induced morphological features of apoptosis in enteric neurons in a concentration-dependent manner. Potentiation of toxicity resulted from co-administration of H_2O_2 and SNP. Pretreatment of cultures with glial cell line-derived neurotrophic factor (GDNF) attenuated H_2O_2-induced neurodegeneration, but not that induced by SNP or combined exposure to H_2O_2 + SNP. This study establishes oxidative stress and lipid peroxidation as mechanisms involved in enteric neurodegeneration and identifies GDNF, a neurotrophin known to promote survival of embryonic enteric neurons, as a neuroprotective agent for adult myenteric neurons in primary culture against specific neurotoxic insults.

Further experiments were performed to determine and characterize the susceptibility of enteric neurons *in vitro* to hypoxia-reoxygenation and anoxia-reoxygenation injury. Although hypoxia-reoxygenation injury has been well characterized in the CNS, in the gut where susceptibility to this type of injury is high, it is

poorly understood. Additionally, little information exists regarding the susceptibility of enteric neurons that control gut motility to hypoxic and anoxic injury. In our study, hypoxia (O_2 tension of 2%) of 1 to 24 hrs duration, followed by prolonged reoxygenation, did not significantly alter the survival of enteric neurons or non-neuronal cells in culture. Anoxia, defined as an O_2 tension < 0.01%, followed by reoxygenation, induced neuronal depletion proportional to the duration of the O_2 deprivation. In contrast to the marked neuronal susceptibility to such damage, enteric glia were highly resistant to anoxia-reoxygenation insult. Both anoxia and subsequent reoxygenation induced neuronal cell death in ENS cultures. Our results indicate that like the CNS, the ENS is highly sensitive to anoxia-reoxygenation and that therapeutic modalities used for anoxic CNS injury represent candidate treatments for enteric nerve damage.

Introduction

The enteric nervous system (ENS) consists of two primary interconnected ganglionated nerve layers, the myenteric and submucous plexuses, which can function independently of the central nervous system to regulate the motility, exocrine and endocrine secretions, immune and inflammatory processes, and microcirculation of the gastrointestinal tract (Gershon et al., 1994; Costa and Brookes, 1994; Furness and Bornstein, 1995; Cooke, 1994; Surprenant, 1994; Lundgren et al., 1989). Alterations in enteric control, axonal damage, and neuronal cell loss lead to disordered or impaired gastrointestinal movement, nausea, vomiting, weight loss and malnutrition characteristic of intestinal disease. The importance of the ENS in gut function and dysfunction has made it the subject of intensive research in recent years (Furness and Costa, 1987a; Wood, 1987; Gershon et al., 1993).

In vitro cultures are useful systems in which to study neurons. The study of neurons *in vitro* permits precise control over experimental parameters and permits direct observation, characterization, and experimental manipulation of specific cell types. Studies on nervous tissue in primary culture have generally been performed using embryonic or neonatal tissues (Scott, 1982), as cells at early developmental stages possess greater potential for growth in culture (Crain, 1976). Previously, both explant cultures (Jessen et al., 1983) and dissociated cell cultures (Nishi and Willard, 1985) from neonatal rat myenteric plexus have been developed for study of the ENS. However, embryonic and neonatal cultures do not represent accurate models of adult enteric neuronal systems, as ENS maturity is reached only at one month after birth. Indeed, certain neuronal populations can only first be detected two to three weeks after birth

(Matini et al., 1997), and nerve cell numbers per ganglia decrease by approximately half in the first two weeks of life (Schafer et al., 1999). Cultured enteric neurons from adult animals are thus required for relevant *in vitro* studies of the mature ENS.

Damage and degeneration of the adult ENS occurs in a variety of gastrointestinal and extra-intestinal diseases via unknown mechanisms. Cultures of the adult ENS represent a highly relevant, simplified system in which neurotoxic mechanisms that potentially underlie clinical damage of the adult ENS can be identified. Adult ENS cultures also represent an ideal system for identification of neurotrophic factors that may protect enteric neurons during neurotoxic diseases. Neuronal populations commonly exhibit varying responses to specific neurotrophic factors dependant upon their stage of development. Thus, adult ENS cultures represent a far more relevant system for identification of neuroprotective factors for the adult ENS *in vivo* than do embryonic and neonatal ENS cultures, which are not fully developed and so may not respond to the same neurotrophic factors. We report here on our development of a dissociated cell culture protocol for enteric neurons isolated from the myenteric plexus of adult rat ileum.

Neurotoxicological studies are commonly performed *in vitro* (Silani et al., 2000; Sawyer, 1995), as rapid and relatively inexpensive tests can be performed in highly reproducible, controlled systems. Indeed, much of the current information on inflammatory damage within the CNS has also been gained by the use of *in vitro* toxicological studies (Snider et al., 1999; Zipfel et al., 1999). To facilitate study of neuropathogenesis within the ENS, we optimized our adult enteric cell cultures for toxicological experimentation. To this end, the culture substrate was optimized to withstand degradation and cell detachment presumably caused by degradative enzymes

frequently released from damaged cells (Colton et al., 1993; Weber and Waghray, 1995). The ability to detect and characterize cell death mechanisms was also tested in these cultures.

Limitations to the use of such primary *in vitro* ENS culture systems for experimentation include the requirement for costly specialized dissection equipment and facilities. Additionally, a significant time input by trained personnel is required for the microscopic dissection of tissue and cell dissociation in this culturing procedure. Finally, these primary cell culture procedures require animals to be available as a tissue souse for use in every experiment; necessitating animal care facilities to be available to the investigator and experiments to be planned far in advance to ensure animals are available for use. These requirements preclude the use of such primary ENS cultures by many laboratories not specialized for such purposes, limit the flexibility of experimental schedules, and hinder collaborations between laboratories.

In order to facilitate our studies using these cultured adult rat enteric neurons, and to enable collaborative work with other laboratories, we developed a proprietary procedure for cryopreservation of dissociated enteric neurons. Using this cryopreservation procedure, dissociated ENS cells isolated by a given laboratory can be frozen and shipped to their destination, where they can be rapidly thawed and cultured in a simple procedure. Cryopreserved cell stocks can also be readily thawed and cultured as required, in only those amounts needed for a given experiment. In this study, the viability of ENS cell populations within cryopreserved cultures was compared with those of freshly-isolated counterparts, and the morphological, neurochemical, and electrophysiological characteristics of cryopreserved neurons determined to assess their

relevance to both the *in vivo* adult ENS and to freshly-plated ENS cultures. Unfortunately, due to the proprietary nature of the cryopreservation protocol, no discussion of the cryopreservation methods used, or their comparison with other published cryopreservation methods can be made in this thesis. With the availability of both freshly-plated and cryopreserved cultures of adult enteric neurons specifically optimized for neurotoxicological experimentation, study of the widely unknown field of enteric neurodegeneration can proceed rapidly.

Enteric neurodegeneration contributes to motor, secretory, inflammatory, and immunologic dysfunction of the gut (Smith, 1982) and is a feature of gut diseases including inflammatory bowel disease (IBD) (Dvorak et al., 1980), infantile hypertrophic pyloric stenosis (Spitz and Kaufmann, 1975), and intestinal pseudoobstruction (Schuffler, 1984). Enteric neurodegeneration also often accompanies diabetes (Buchan, 1990), myotonic dystrophy (Yoshida et al., 1988), and CNS diseases such as Parkinson's (Foley and Riederer, 2000). Animal models have also shown enteric neurodegeneration to be rapidly induced during intestinal inflammation (Sanovic et al., 1999). However, the mechanisms underlying enteric neurodegeneration are not known.

In the CNS, reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) and nitric oxide (NO) have been implicated as mediators of neuronal apoptosis (Bredesen, 1995), and are believed to play important roles in the neurodegeneration characteristic of Alzheimer's disease, Parkinson's disease, and stroke (Mattson et al., 1996; Simonian and Coyle, 1996; Dexter et al., 1994). ROS production is also enhanced during experimental IBD, and is thought to mediate some of the associated damage (Grisham et al., 1994; Herulf et al., 1998). It has recently been reported that glutamate,

which induces CNS neuronal cell death via mechanisms including oxidative stress (Verity, 1993), produces neurotoxicity in enteric neurons (Kirchgessner et al., 1997). Furthermore, NO inhibition attenuates the disruption of myenteric ganglia in a rat model of colitis (Hogaboam et al., 1995). We thus hypothesized that oxidative stress mechanisms are involved in enteric neurodegeneration.

The pathological consequences and underlying mechanisms of ischemia-reperfusion injury are well established with regard to the central nervous system (CNS) (Podolsky, 1992). Ischemia-reperfusion injury is also very important but much less well studied in the gastrointestinal (GI) tract, where it produces a spectrum of pathologic changes that range from reversible mucosal injury to transmural necrosis (Mitsudo and Brandt, 1992).

Within the GI tract, ischemia-reperfusion and hypoxia-reoxygenation injury ensue from a variety of causes, including radiation enteritis, trauma, chemotherapy, and radiation (MacCannell, 1993; Rha et al., 2000). The most common known causes of such injury include inflammatory bowel diseases (such as ischemic colitis, ulcerative colitis, and Crohn's disease), arterial occlusion, and post-operative obstruction of the gut (Osborne et al., 1993; Knudsen et al., 1998; Alpati and Mihas, 1999). Ischemia-reperfusion and hypoxia-reoxygenation injury in the GI tract have extensive potential incidence due to the widespread occurrence of the conditions and diseases which cause such injury. For example, approximately 6000 new cases a year of Crohn's disease are reported in the United States, with a disease incidence estimated to be 30-200 cases per 100,000 persons (Calkins and Mendoloff, 1995). Between 1300 and 16,500 cases of ulcerative colitis are reported per year in the U. S., with this disease occurring in 1.2-106

people per 100,000 (Calkins and Mendoloff, 1995). Furthermore, the post-operative incidence of bowel obstruction is also high, occurring in more than 10% of patients following abdominal surgery (Charles and Bail, 1993). The pathophysiological consequences of intestinal ischemia are likely to involve cellular hypoxia, hypercarbia, and associated metabolic changes (Knudsen et al., 1998).

Neurodegenerative changes have been observed in the ENS of patients likely to have hypoxia/ischemia injury; those with arterial occlusions, post-operative ischemic damage, Crohn's disease and ulcerative colitis (Oehmichen and Reifferscheid, 1977; Dvorak et al., 1980; Steinhoff et al., 1988; Lindgren et al., 1991). This ENS damage produces clinical symptoms including disturbed gastrointestinal transit, reduced peristalsis, disordered small bowel movement, atony of the large bowel, nausea, vomiting, and in advanced stages, weight loss and malnutrition (Debas and Mulvihill, 1991). *In vivo* studies have shown an association between intestinal ischemia and morphological changes in the myenteric plexus of rat intestine (Yano et al., 1997). From these findings, it is hypothesized that intestinal ischemia may induce ganglion cell atrophy and thus affect intestinal peristalsis. However, it is unclear whether hypoxia/ischemia or the frequently associated intestinal inflammation injures the ENS during disease. In contrast to the extensive knowledge in the CNS, investigation of hypoxic injury within the ENS remains to be undertaken. In this initial study, we sought to determine the susceptibility of enteric neurons to hypoxia-reoxygenation and anoxia-reoxygenation insult, and to characterize these damage processes.

Glial cell line-derived neurotrophic factor (GDNF) acts as a survival factor for many classes of sensory and autonomic neurons (Buj-Bello et al., 1995) by suppression

of apoptotic cell death (Clarkson et al., 1995; 1997). In adult tissues, GDNF has been shown to protect brainstem dopamine neurons and motorneurons in a variety of injury models (Beck et al., 1995; Bowenkamp et al., 1995; Tomac et al. 1995, Li et al., 1995). GDNF and its receptor Ret are also required for the development of the enteric nervous system (ENS) (Sanchez et al., 1996; Schuchardt et al., 1994); stimulating proliferation of migratory enteric neural precursor cells and their differentiation into neurons (Hearn et al., 1998; Heuckeroth et al., 1998). The function of GDNF in the mature ENS is not known but, in the adult, enteric GDNF concentrations remain high and its receptor Ret is expressed in adult myenteric neurons (Peters et al., 1998). We hypothesize that, similar to its neuroprotective role in adult CNS and autonomic neurons, GDNF is involved in the neuroprotection and maintenance of adult enteric neurons.

We present here a study of the roles of oxidative stress, lipid peroxidation, and hypoxic processes in enteric neurodegeneration and the neuroprotective effects of GDNF using an *in vitro* culture model of adult rat myenteric neurons. Our aim was to evaluate the sensitivity of cultured myenteric neurons to agents that mediate lipid peroxidation, oxidative stress, free radical injury, and anoxia-reoxygenation. The ability of the neurotrophin GDNF to act as a neuroprotective agent against such damage in enteric neurons was also investigated.

Materials & Methods

ENS Tissue Isolation

For cultures of dissociated cells from the myenteric plexus of the distal ileum of the adult rat, laminar sections of myenteric plexus-longitudinal muscle are first isolated by dissection, then this tissue is dissociated into individual cells for culture. Male Sprague Dawley rats 200-250g (Charles River laboratories, Canada) were sacrificed by cervical dislocation according to Canadian Council on Animal Care guidelines, and a 30 cm segment of distal ileum was isolated. This tissue was flushed with freshly-prepared oxygenated Kreb's solution (7.36 g/L NaCl, 0.385 g/L KCl, 0.166 g/L $NaH_2PO_4(H_2O)$, 0.245 g/L $MgCl_2.6H_20$, 0.368 g/L $CaCl_2.2H_2O$, 2.0 g/L glucose, and 2.1 g/L $NaHCO_3$) to remove intestinal contents, and tissue was maintained in continuously-oxygenated Kreb's solution on ice until processed for dissection. Two cm segments of ileum were cut longitudinally and pinned flat, mucosal side up. Throughout dissection, tissue was maintained in continuously oxygenated Kreb's solution. Under a dissecting microscope, villi were scraped away from underlying muscle layers. Mucosa and circular muscle, as a single layer, were then separated from the myenteric plexus and longitudinal muscle layers underneath. Remaining circular muscle fibres were removed. The resulting 'laminar preparations' were maintained on ice until processed for enzymatic dissociation of cells.

Enzymatic Dissociation of ENS Cells

Following dissection, isolated laminar preparations of myenteric plexus/longitudinal muscle were mechanically and enzymatically dissociated to obtain single cells for culture. Isolated tissue was placed in 10 ml of filter-sterilized Hanks

Balanced Salt Solution pH 7.3 (Gibco BRL) containing 100U of papain II (Worthington) and 1.0 mg of L-cysteine (Sigma), and then incubated for 10 min. in a 37°C water bath. This tissue was rinsed in a L-15 washing medium composed of Leibovitz's L-15 medium (Gibco BRL) with 10% fetal bovine serum (Gibco BRL), 2mM L-glutamine (Gibco BRL), 15 mM glucose (BDH) and 100U penicillin-streptomycin (Gibco BRL). After rinsing, tissue was incubated in a 37°C water bath for 10 min. in 10 ml of Hanks Balanced Salt Solution (Gibco BRL) containing 3000U of collagenase type I (Worthington) and 20U of dispase II (Boehringer Mannheim). Tissue in this solution was then triturated with a flame-polished Pasteur pipette until cells were dissociated (approximately 40 triturations). This medium was centrifuged at 4°C for 5 min. at 500 rpm, and the pellet resuspended in 8ml of L-15 washing medium. Centrifugation was repeated for 5 min. at 500 rpm and 4°C, and the pellet was resuspended in feeding medium composed of 90% MEM (Gibco BRL), 2.5% rat serum (Gibco BRL), 2 mM L-glutamine, 15 mM glucose, 100U penicillin-streptomycin (Gibco BRL), 10 μM fluorodeoxy-uridine, 1 mM cytosine arabinoside and 0.5 mM uridine triphosphate.

ENS Culture Substrate Selection

A variety of culture substrates were tested to determine the optimal substrate for ENS neurotoxicological experimentation. Substrates must optimally promote cell attachment and viability, as well as be able to withstand degradation and prevent cell detachment upon toxic insult. Culture substrates tested for use in neurotoxicological experiments included 0.1 mg/ml poly-d-lysine alone or in combination with 20 μg/ml laminin or 10%, 20%, 30%, 40% or 50% collagen, plated according to standard protocols (McKeehan and Ham, 1976). Rat tail collagen and Matrigel matrix (VWR Canlab) were

also tested as culture substrates, and were applied onto culture wells according to manufacturer's instructions. Selection of an optimal substrate was based upon adherence and survival of neurons within culture, as quantitated after 7 days *in vitro*, and the ability of substrates to withstand degradation during toxic insult (24 hr exposure of 7 day ENS cultures to 0.01 mM - 1 mM H_2O_2). Neuronal adherence and survival was determined for 7 day cultures in which total neuronal numbers present in culture wells were quantitated. Substrate susceptibility to degradation during toxic insult was characterized by marked sloughing of large sheets of morphologically normal cells away from the culture well surface in response to toxic insult. Within cultures plated upon susceptible substrates, the vast majority of cells present in culture were lost.

Cell Plating Of The Dissociated ENS

Once dissociated cells were obtained from adult ENS tissues, cells were plated at defined concentrations for extended culture. Cell suspensions were plated at an approximate concentration of 570 cells / mm^2 onto sterile glass LabTek 8 well chamber slides (VWR Canlab) coated with Matrigel matrix (VWR Canlab). Cultures were maintained at 37°C in an incubation chamber (gassed with 5% CO_2 in air), and feeding medium was changed after the initial 24 hr incubation and every 72 hrs subsequently. Cultures were generally used in experiments seven days after plating, at which point cultures were both mature and stable.

Cryopreservation Of Dissociated ENS Cells

Enteric neurons destined for cryopreservation were dissected and dissociated following procedures identical to those used for cells destined for immediate culture. However, instead of plating, these dissociated cells were cryopreserved using a

proprietary protocol (QBM Cell Science, Ottawa, Canada). Following seven days cryopreservation, cells were thawed and plated according to that cell plating protocol used for freshly dissociated ENS cells. Cryopreserved cells were cultured according to standard ENS protocols. For evaluative purposes, dissociated cells from a single animal were divided equally for immediate plating and for cryopreservation. These sister cultures were used for direct comparison of freshly plated and cryopreserved cell cultures from the same animal.

Cortical Cell Cultures

Due to our unfamiliarity with the use of hypoxia chambers for experimentation, cortical cell cultures with known responses to hypoxic/anoxic insults were used as positive controls to verify our correct use of hypoxia chambers for our ENS toxicity experiments. For these controls, mixed neocortical cultures, containing both neurons and glia, were prepared from fetal rats at 19 days gestation, as generally described previously for murine neocortical cultures (Choi et al., 1987). Dissociated cells were plated in a MEM-based plating medium containing 20mM glucose, 2mM glutamine, and 10% heat inactivated horse serum. Cultures were fed twice weekly with this medium and maintained at 37°C in a humidified incubator (gassed with 5% CO_2 in air). Cultures were used for experimentation fourteen days after plating.

Live Culture Observation

Enteric cell cultures, including both freshly plated and cryopreserved cultures, were observed microscopically (Zeiss Axiovert S100 TV) upon plating and every subsequent 24 hrs to monitor the progression of cell adhesion to culture substrate and process extension. Cultures were removed from their incubator for no longer than 10

min. at a time for observation. Images were captured digitally using a 10 bit digital camera (DVC, Austin, TX) and Northern Eclipse image-capture software (Toronto, Canada).

Immunohistochemical Characterization Of Freshly Plated And Cryopreserved ENS Cultures

Seven day ENS cultures, selected for use in neurotoxicological experiments, were characterized to determine both their cellular composition and their relevance to the *in vivo* ENS. To that end, the component cell types within ENS cultures were identified, and the neurochemical, morphological and electrophysiological characteristics of cultured neurons determined. Seven day cell cultures of freshly plated and cryopreserved neurons were fixed for 60 minutes in a modified Zamboni's fixative consisting of 4% paraformaldehyde and 0.4% picric acid in 0.16 M phosphate buffer (PB), pH 6.9. Fixative was cleared with several rinses in 0.1M phosphate buffered saline (PBS) prior to immunohistochemical or histochemical staining. 1 cm tissue segments were also isolated from the adult rat ileum, and fixed by identical means. Following overnight incubation in a 20% sucrose solution, this tissue was cryostat sectioned to produce 14 µM thick sections. These tissue sections were used as positive controls in all immunohistochemical staining procedures.

Immunohistochemical staining for identification of cell types and specific neuronal subtypes within cultures was performed using established commercially available antibodies. The same general procedures were followed for all antibodies used. Primary antibody diluted to working concentration was applied to culture slides overnight at 4°C. After rinsing in 10 mM phosphate-buffered saline (PBS), secondary antibody at

its appropriate working dilution was applied to slides for 35 min. at 37°C. Slides were again rinsed in PBS, then mounted under a fluorescence fade-retarding mounting medium (1 mg/ml p-phenylenediamine in 0.9% NaCl, 10% glycerol).

Morphological identification of cell types present in cultures was confirmed immunohistochemically using cell-type-specific antibodies. Polyclonal anti-neuron-specific enolase (NSE; 1:400 dilution; Dako) and Cy3-conjugated donkey anti-rabbit (1:100; Jackson) were used to visualize enteric neurons. Polyclonal anti-glial fibrillary acidic protein (GFAP; 1:400; DakoPatts) and FITC-conjugated donkey anti-rabbit (1:80; Chemicon) were used to visualize enteric glia. Monoclonal anti-vimentin (1:20; DakoPatts) and Cy3-conjugated sheep anti-mouse (1:100; Sigma) were used to visualize glia and fibroblasts. Double staining using anti-GFAP and and-vimentin was used to distinguish enteric glia, which positively stain for both GFAP and vimentin, from fibroblasts, which positively stain exclusively for vimentin. Cultures double-stained to distinguish glia and fibroblasts were used for quantitative determination of the proportions of glia and fibroblasts that constitute the non-neuronal cell population present in ENS cultures. Monoclonal anti-smooth muscle actin (1:100), a gift of Dr. M. McBurney (Rudnicki et al., 1990) and FITC-conjugated goat anti-mouse (1:80; American Qualex) were used to visualize smooth muscle cells. Polyclonal anti-macrophage (1:400; Accurate) and FITC-conjugated donkey anti-rabbit (1:20 dilution; Amersham) were used to visualize macrophages. Polyclonal anti-histamine (1:200; Incstar) and FITC-conjugated donkey anti-rabbit (1:20; Amersham) were used to visualize mast cells, gut endocrine cells and blood cells. Monoclonal anti-c-kit (1:200; Santa Cruz) and FITC-

conjugated goat anti-mouse (1:20; American Qualex) were used to visualize interstitial cells of Cajal.

Antibody staining to characterize enteric neuronal subtypes present in cultures was also performed. Polyclonal anti-nitric oxide synthase (NOS) (1:400; Santa Cruz) and Cy3-conjugated donkey anti-rabbit (1:100; Jackson) were used to visualize NOS neurons. Polyclonal anti-vasoactive intestinal polypeptide (VIP) (1:400; Peninsula) and Cy3-conjugated donkey anti-rabbit (1:100; Jackson) were used to visualize VIP in neurons. Polyclonal anti-met-enkephalin (Met-Enk) (1:500), a gift of Dr. R. Elde (Giesler and Elde, 1985), and Cy3-conjugated donkey anti-rabbit (1:100; Jackson) were used to visualize enkephalin in neurons. Polyclonal anti-gamma aminobutyric acid transaminase (GABA-T) (1:400) (Nichols et al., 1995) and Cy3-conjugated donkey anti-rabbit (1:100; Jackson) were used to visualize GABA transaminase within neurons. Monoclonal anti-somatostatin (SOM) (1:100), a gift of Dr. S. Vincent (Mizukawa et al., 1988), and FITC-conjugated goat anti-mouse (1:20; American Qualex) were used to visualize somatostatin in neurons. Polyclonal anti-substance P (1:2000; Peninsula), and Cy3-conjugated donkey anti-rabbit (1:100; Jackson) were used to visualize substance P immunoreactivity within neurons. For quantitation of Dogiel/Stach neuronal morphological subtype composition, polyclonal anti-PGP 9.5 (1:400; Chemicon) and FITC-conjugated donkey anti-rabbit (1:20; Amersham) were used. For double staining procedures using two polyclonal rabbit antibodies, the double label Fab antibody kit (Jackson ImmunoResearch) was employed.

Quantitation And Analysis Of Cell Types And Neuronal Subtypes Within Freshly-Plated And Cryopreserved ENS Cultures

Percentages of component cell types in ENS cultures were quantitated, as were those of component neuronal subtypes. Such quantitation contributes to the characterization of these cultures and permits their comparison with the *in vivo* ENS. All cellular quantitation was performed in 7 day ENS cultures unless otherwise indicated. Manual counts were performed for all quantitation of cell populations. For all cellular quantitation, all cells of interest present within an experimental culture well were counted, unless otherwise indicated. Data were expressed as the mean value $\pm$ standard deviation for these n=5 experimental wells. Within culture wells, neurons were visualized under fluorescent conditions with anti-NSE antibody, and neuronal numbers in each culture well were counted manually. Total cell numbers, as indicated by Hoechst 33258 histochemical staining of all cell nuclei, were also counted manually. Immunohistochemically-identified neuronal subtypes were also counted manually and compared to total neuronal numbers within the same well. The respective percentages of glia and fibroblasts, which constitute those non-neuronal cells present in culture, were also determined. The total number of glial cells, those that express both anti-GFAP immunoreactivity and anti-vimentin immunoreactivity, were counted manually in culture wells. Total numbers of fibroblasts, as indicated by positive staining for vimentin and negative staining for GFAP, were also counted manually in these same culture wells. The composition of non-neuronal cells in culture was then determined. Glial percentages of non-neuronal cells were calculated by determining the percentage of vimentin-positive cells that were also GFAP-positive. Fibroblast percentages of non-neuronal cells were

calculated by determining the percentage of vimentin-positive cells that were GFAP-negative. For quantitative assessment of the percentage of total neurons that express a specific neurotransmitter, experimental culture wells were double-stained with anti-NSE antibody and an antibody raised against a specific neurotransmitter. The total number of neurons immunopositive for that specific neurotransmitter was then determined and expressed as a percentage of total neurons present in that experimental well. For quantitation of the Dogiel/Stach type neuronal morphologies present in culture, the morphological subtypes of 100 anti-PGP 9.5 stained neurons were identified.

Histochemical Evaluation of Nitric Oxide Synthase Activity with ENS Cultures

Histochemical methods were employed to determine the levels of nitric oxide synthase (NOS) enzyme activity in ENS cultures. NADPH-diaphorase histochemical staining for NOS activity within cultures was performed according to published methods (Nichols et al., 1994). Fixed cultures were rinsed in 10 mM PBS, NO synthase reaction medium [1 mM NADPH, 0.5 mM nitroblue tetrazolium, 0.3% Triton X-100 in 10 mM PB (pH 8.0)] was applied, and culture slides incubated in a dark, moist chamber at 37°C for 1 hr. Slides were then rinsed in 10 mM PBS for 15 min., and in decreasing concentrations of 100 mM PB, 50 mM PB, 25 mM PB, and 12.5 mM PB for 1 min. each. They were then air-dried and coverslipped under Tris-Glycerol 1:3. The histochemical product for NADPH-dependent diaphorase, indicating NOS activity, was photographed under bright-field conditions. Grayscale value quantitation of the intensity of NADPH diaphorase staining, indicative of the levels of NOS activity within cells, was performed using UTHSCA ImageTool (version 2.00). Increasing grayscale values are indicative of decreasing staining intensity.

Electrophysiology Of Freshly-Plated And Cryopreserved Neurons

Electrophysiological recording of cultured enteric neurons was assayed to determine the feasibility of such *in vitro* experimentation, as well as for culture characterization. All electrical recordings were performed using an Axopatch 200A amplifier attached to a Digidata 1200 A/D interface and controlled by pClamp software. Signals were lowpass filtered at 2 kHz before being digitized at 10 kHz and saved to the hard disk of a PC computer, or digitized at 20 kHz and saved on a DAT tape recorder. Patch pipettes were fabricated from 1.5 mm outer diameter, thin-wall, borosilicate glass (World Precision Instruments, Sarasota, FL) and typically had resistances of 3-6 MΩ when filled with the electrolyte solution described below. Series resistance in the whole-cell configuration was 8-25 MΩ of which 80% was routinely compensated. The series resistance was checked periodically and recordings were abandoned if the resistance rose irreversibly above 25 MΩ. Neuronal input resistance was determined by measuring the steady state current responses to voltage steps of -10 mV from a holding potential of -60 mV. External solution for electrical recordings was composed of (in mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 mM glucose, and 10 mM HEPES (300 mOsm, pH adjusted to 7.3 -7.4). Electrode solution for filling patch pipettes consisted of (in mM) KCl 145; 10 NaCl; 10 HEPES; 1 Na_2EDTA; and glucose 20; adjusted to pH 7.2-7.3. Osmolarity of the pipette solution was adjusted as required by addition of distilled water or sucrose. Neurons selected for electrophysiological recording were those with the gross morphological appearance of Dogiel type II neurons.

Identification of Cell Death Mechanisms Within ENS Cultures

To identify and characterize in vitro neurotoxicity, cell death and cell death mechanisms must be readily identifiable in experimental cultures. In ENS cultures, initial toxic insults were given expressly to evaluate death mechanism identification methods in these cultures. Toxicological tests were performed using 0.01 mM-1 mM hydrogen peroxide (H_2O_2) added to 7 day cell cultures for 24 hrs prior to fixation. These toxic insults were used for the evaluation of substrate resistance to degradation during toxicological insult, and to determine if cell death could be identified and characterized within our cultures.

Apoptosis was identified in enteric cultures using Hoechst 33258 histochemistry, annexin V/propridium iodide, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining methods according to established protocols. All chemicals for TUNEL reactions were purchased from Boehringer Mannheim, Canada, those for annexin V from R & D Systems, Canada, while Hoechst 33258 was purchased from Sigma, Canada. Necrosis was visualized using Fluoro-Jade (Histo-Chem, Inc., Virginia) following the procedure of Schmued et al. (1997).

Quantitation Of ENS Toxicity

Quantitative determination of cell type specific toxicity was required in ENS culture experiments. For such quantitation, cultured neurons were visualized under fluorescent conditions with anti-NSE antibody, and neuronal numbers in each culture well were counted manually. Neurons actively undergoing apoptosis, as indicated by Hoechst 33258 staining, were deducted from numbers of surviving neurons. Toxicity was expressed in terms of cell survival, and compared to that of controls. Total cell numbers

were counted manually by means of Hoechst 33258 staining of all cell nuclei, excluding visibly apoptotic and necrotic nuclei from viable cell counts. Non-neuronal cell numbers were determined as total surviving cell counts less surviving neuronal counts.

Chemical Insult Of ENS Cultures

The toxicity of several known reactive oxygen species was tested in our ENS cultures. In these experiments, seven day cell cultures were exposed to varying concentrations of toxins [4-hydroxynonenal (HNE Sigma, Canada) 25, 50, 100, 200 μM; hydrogen peroxide (H_2O_2, Sigma, Canada) 1, 10, 100 μM, 1 mM; menadione (Sigma, Canada) 1, 10, 100 μM; sodium nitroprusside (SNP, Sigma, Canada) 0.1, 0.5, 1 mM] diluted in feeding medium for 24 hrs. Similar experiments were performed involving the treatment of cultures with combinations of H_2O_2 and SNP (0.5 mM SNP + 1 μM H_2O_2; 0.5 mM SNP + 10 μM H_2O_2). Following toxin exposure, cultures were rinsed in fresh feeding medium, and then fixed for 60 minutes in a modified Zamboni's fixative consisting of 4% paraformaldehyde and 0.4% picric acid in 0.16 M phosphate buffer (PB), pH 6.9. Fixative was cleared with several rinses in 0.1 M phosphate buffered saline (PBS) prior to immunohistochemical or histochemical staining.

Hypoxic/Anoxic Insult Of ENS Cultures

To determine the neurotoxicity of hypoxia-reoxygenation and anoxia-reoxygenation insults, cortical and enteric neuronal cultures were exposed to either 2% O_2 (hypoxia) or <0.01% O_2 (anoxia) in a 37°C chamber gassed with either CO_2 3%, O_2 2%, H_2 5%, N_2 90% or CO_2 5%, H_2 5%, N_2 90%, respectively. Cultures were maintained in these experimental conditions, for either 1, 3, 6, or 24 hrs, after which they were transferred to normoxic conditions in a 37°C incubation chamber (gassed with 5% CO_2 in

air), and were allowed to slowly reoxygenate without changing the medium. Twenty-four hours following initiation of the experiment, cultures were removed from the chamber and fixed for 60 minutes in a modified Zamboni's fixative consisting of 4% paraformaldehyde and 0.4% picric acid in 0.16 M phosphate buffer (PB), pH 6.9. Fixative was cleared with several rinses in 0.1 M phosphate buffered saline (PBS) prior to immunohistochemical or histochemical staining. For these experiments, cortical neuron cultures (E19 rat cortical cells tested at 14 days *in vitro*) were used as positive controls.

For determination of the individual roles of anoxia and reoxygenation in enteric neurotoxicity, enteric cultures were exposed for a given time period to either anoxia or anoxia-reoxygenation. Following the general experimental procedure detailed previously, cultures were exposed to anoxia for either 3 or 5 hrs, and to anoxia-reoxygenation for either 3 hrs total (1 hr anoxia followed by 2 hrs reoxygenation) or for 5 hrs total (3 hrs anoxia followed by 2 hrs reoxygenation). At the end of these time periods, cultures were fixed for histochemical and immunohistochemical staining.

GDNF Neuroprotection Against Toxic Insult

Glial cell line-derived neurotrophic factor (GDNF) was tested as a neuroprotective factor against specific neurotoxic insults to the ENS. GDNF (10 ng/ml, diluted in PBS, R & D Systems, Canada) was added to feeding medium of 7 day cell cultures 24 hrs prior to addition of toxin, as in previous culture systems (Clarkson et al., 1995; 1997; Sawada et al., 2000). The chemical toxins added were 100 μM H_2O_2, 1 mM SNP, and 1 μM H_2O_2 + 0.5 mM SNP, as these produced comparable (approximately 50%) neurotoxicity in ENS cultures. GDNF's ability to protect against the neurotoxic

effects of 1 hr anoxia followed by 23 hr reoxygenation, which also induced approximately 50% neurodegeneration in ENS cultures, was also tested. Cultures were first incubated with GDNF for 24 hrs, then exposed to toxic insults for an additional 24 hrs in the presence of GDNF. ENS cultures were then were fixed and stained immunohistochemically as described previously. For each experimental culture well, controls were exposed only to GDNF for 48 hrs, to toxic insult for 24 hrs, or were kept in feeding medium until fixation.

Statistics

Unless otherwise noted, data are expressed as the mean of n=5 independent experiments. Significant differences in survival between multiple cell groups were determined using one-way ANOVA followed by post-hoc Tukey/Kramer analysis. Significant differences between two groups were determined using two-tailed Student's *t* tests. Significance was considered established at p values of less than 0.01.

For the statistical evaluation of neuronal and total cell populations in cultures of cryopreserved ENS cells, viability was expressed as a percentage of control values, i.e. cell counts from sister cultures which had not been cryopreserved. Data represent the mean of n=5 independent experiments with significant differences between experimental and control values determined using two-tailed student's t test. Significance was considered established at p values of less than 0.01.

Chemicals

Unless otherwise noted, all chemicals were purchased from Sigma, Canada.

Results

Selection Of An Optimal Culture Substrate

The collagen substrate originally used for culture growth was determined to be unsuitable for *in vitro* toxicological experimentation. Marked cell sloughing occurred within cultures in response to toxic insult and this prevented representative and quantitative evaluation of toxicity in these ENS cultures. Substrates determined to be resistant to such toxic degradation and cell sloughing included poly-d-lysine, poly-d-lysine/laminin, poly-d-lysine/collagen, and Matrigel matrix. These substrates were tested in subsequent experiments for their ability to promote neuronal adherence and survival in culture. Neuronal survival upon poly-d-lysine, poly-d-lysine/laminin and poly-d-lysine/collagen substrates was significantly less than that upon collagen substrate. However, neuronal survival upon Matrigel matrix substrate did not significantly differ from that upon collagen substrate (Figure 9).

ENS Culture viability

Upon plating, uniform rounded cells devoid of processes were commonly observed suspended within the culture feeding medium, while small aggregates of viable cells were attached to the culture substrate (Figure 10A). At this time point, cell types were generally difficult to distinguish. However, some glial cells could be identified by their flattened appearance. Although the majority of cells continued to exhibit a uniform rounded appearance after 24 hrs *in vitro*, a small number of cells had extended short processes (Figure 10B). By 48 hrs, cells were flattened upon the culture substrate and demonstrated cell-type characteristic morphologies. Neurons were generally clustered together, and commonly extended several branching processes which intersected with

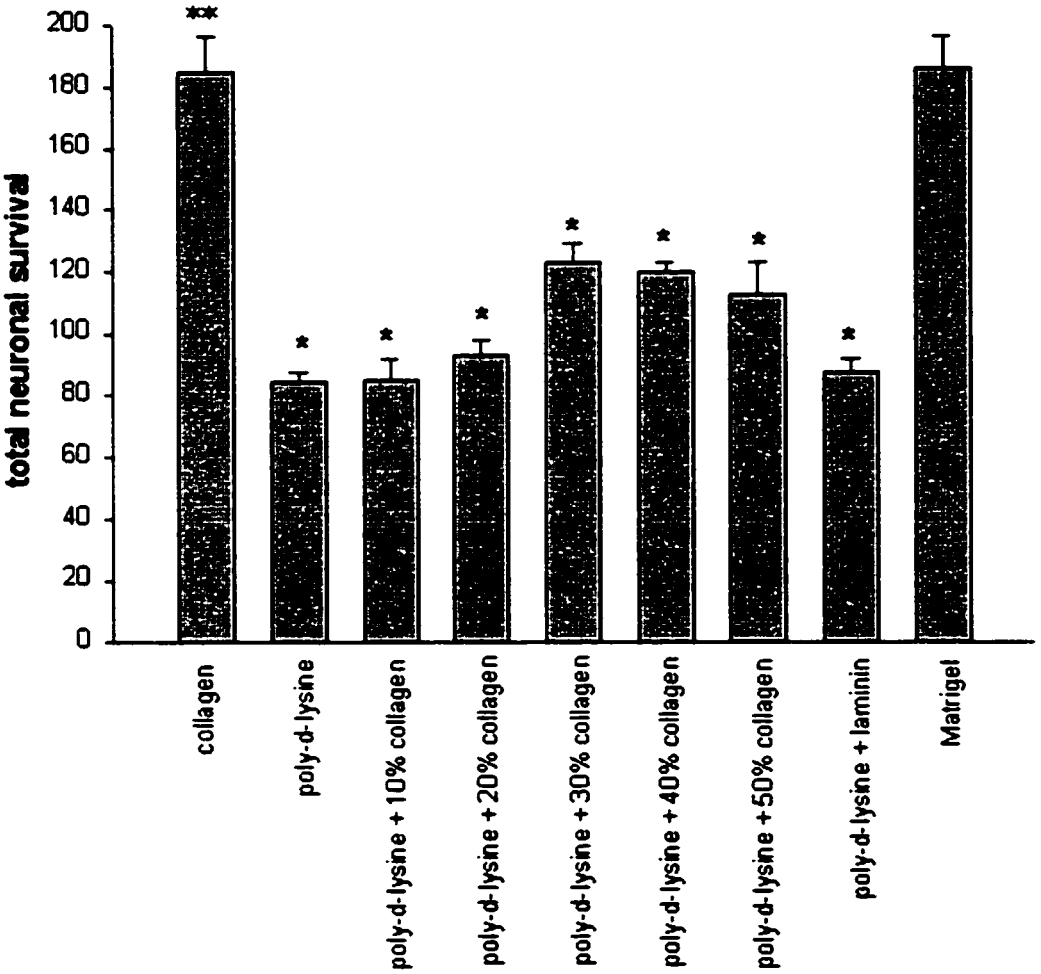

total neuronal survival
200
180
160
140
120
100
80
60
40
20
0
**
*
*
*
*
*
*
*
collagen
poly-d-lysine
poly-d-lysine + 10% collagen
poly-d-lysine + 20% collagen
poly-d-lysine + 30% collagen
poly-d-lysine + 40% collagen
poly-d-lysine + 50% collagen
poly-d-lysine + laminin
Matrigel

Figure 9. Evaluation of ENS culture substrates. Candidate substrates for ENS cultures were tested for their abilities to promote neuronal adherence and survival, as well as for their ability to withstand disruption and degradation upon neurotoxicological insult. Total neuronal numbers in 7 day ENS cultures were counted in n=5 experiments. Substrate disruption and degradation was evaluated upon 24 hr exposure to 0.01mM – 1mM H_2O_2, and identified by observation of morphologically normal cell detachment from the culture plating surface in large sheets. ** indicates that disruption and degradation of the specific substrate tested was observed in response to experimental toxic insult. * indicates those substrates for which total neuronal survival differs significantly from those of cultures plated on collagen substrate (Student's *t* test, $p<0.01$). Whereas neuronal survival does not differ significantly between collagen- and Matrigel-coated culture wells, the ability of Matrigel to withstand toxic insult was responsible for its selection as that substrate used for neurotoxicological experimentation.

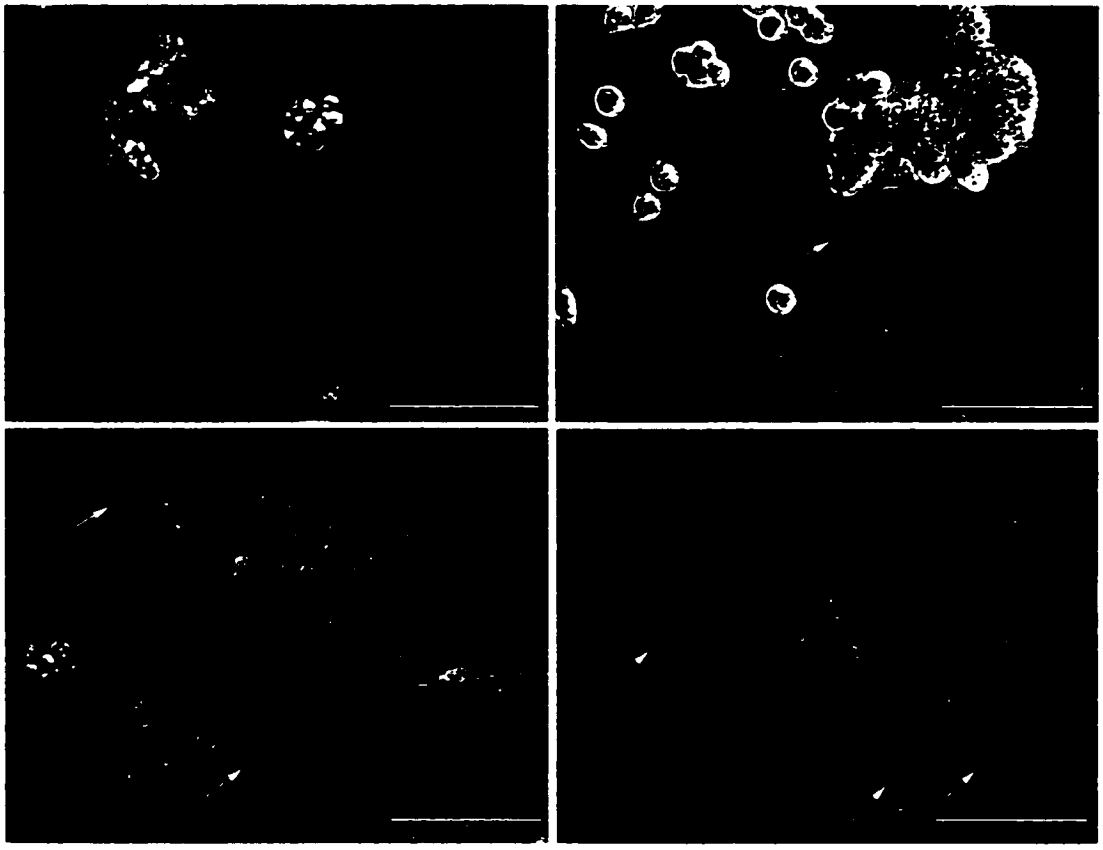


Figure 10. Time course for morphological differentiation of cells in culture. Live cultures of adult rat enteric neurons were characterized over their incubation time course. Upon plating (**A**), cells exhibited uniform rounded appearances. At 24 hrs (**B**), cells had attached to the culture substrate and commenced extending processes. By 48 hrs (**C**), cells had flattened onto the culture substrate, extended considerable branching processes, and exhibited characteristic cell-type morphologies. At 7 days (**D**) cell aggregates resembled *in vivo* myenteric ganglia and extended vast networks of interconnecting processes. Arrows indicate extended processes. Bars represent 100 microns.

many other cell bodies and/or processes within the same cluster or in neighboring neuronal clusters. Neurons preferentially adhered to and overlaid sheets of glial cells, which exhibited characteristic flattened cell bodies with thin membranous expansions and a few processes (Figure 10C). Cultured cells continued to extend branching processes to form complex networks of interconnecting ganglia-like structures by 7 days in culture (Figure 10D).

Immunohistochemical staining was performed to identify the cell types present within cultures. Neuron-specific enolase (NSE), a distinct form of the glycolytic enzyme enolase expressed in most differentiated neurons (Schmechel and Marangos, 1983), identified groups of neurons with interconnecting branched processes within cultures (Figure 11A). Neurons constituted 13.68% $\pm$ 2.23% of total cell numbers (n=5 experiments in which 9237 total cells were counted). All enteric glia contain the intermediate filament protein GFAP (Jessen and Mirsky, 1980; 1983; Jessen et al., 1984). Anti-GFAP staining in our cultures identified large sheets of glia upon which enteric neurons were commonly positioned (Fig. 11B). Double staining for GFAP and the mesenchymal intermediate filament protein vimentin distinguished glia from fibroblasts, as glia were immunopositive for both GFAP and vimentin, whereas fibroblasts were immunopositive exclusively for vimentin. Whereas large numbers of glia were present, only small numbers of fibroblasts were present (Fig. 11C & 11C'). Of all non-neuronal cells present (which represent approximately 86% of the total cell population in cultures), glia represent 83.8% $\pm$ 2.95%, and fibroblasts constitute 16.2% $\pm$ 2.95% (n=5 experiments in which 7851 total non-neuronal cells were counted). Smooth muscle cells, lymphocytes, macrophages, mast cells, endocrine cells, and interstitial cells of Cajal were

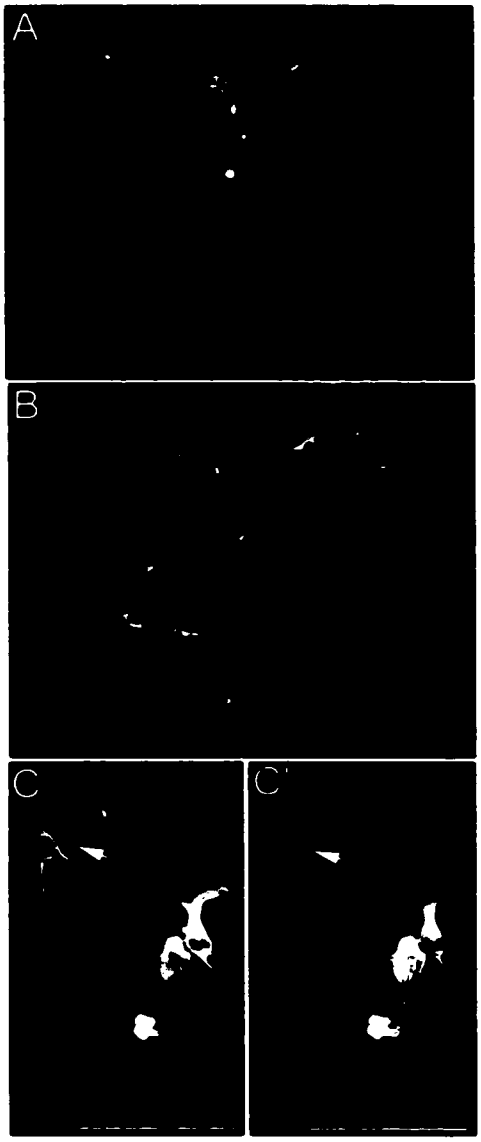
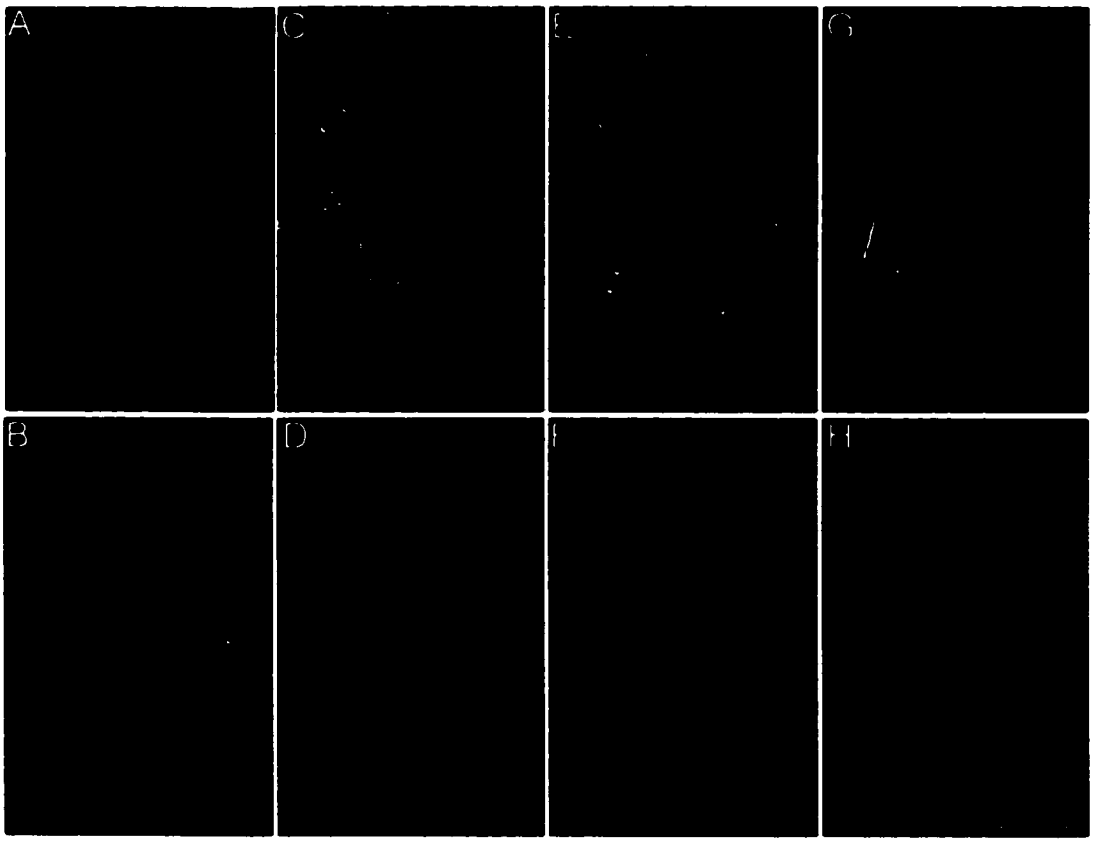

A
B
C
C'

Figure 11. Immunohistochemical identification of cell types present in culture. Fixed 7 day cell cultures were immunohistochemically stained for cell-type identification. (**A**) Neurons and their processes are positively stained for the presence of neuron-specific enolase. (**B**) Anti-GFAP antibody staining identifies glial cells in culture. Double staining for GFAP and vimentin distinguishes glia and fibroblasts. Both fibroblasts and glia positively stain for vimentin (C), but only glia are immunopositive for GFAP (C'). Those cells, positively stained for vimentin but not GFAP, can be identified as fibroblasts (indicated by arrows). Bars represent 100 microns.


A
C
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G
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H

Figure 12. Intestinal cell types not present within enteric neuronal culture. Fibres stained for smooth muscle actin identify circular and longitudinal muscle tissue *in vivo* (**A**). No smooth muscle actin immunoreactivity is present in culture (**B**). Anti-macrophage antibody staining positively labels macrophages within rat ileum (**C**). No macrophages are identified within cultures (**D**). Anti-histamine antibody identifies mast cells, gut endocrine cells, and blood cells *in vivo* (**E**). These cell types are not present in culture, however. (**F**). Finally, anti-c-kit antibody identifies interstitial cells of Cajal present in ileal tissue (**G**), but not in culture (**H**). Bars represent 100 microns.

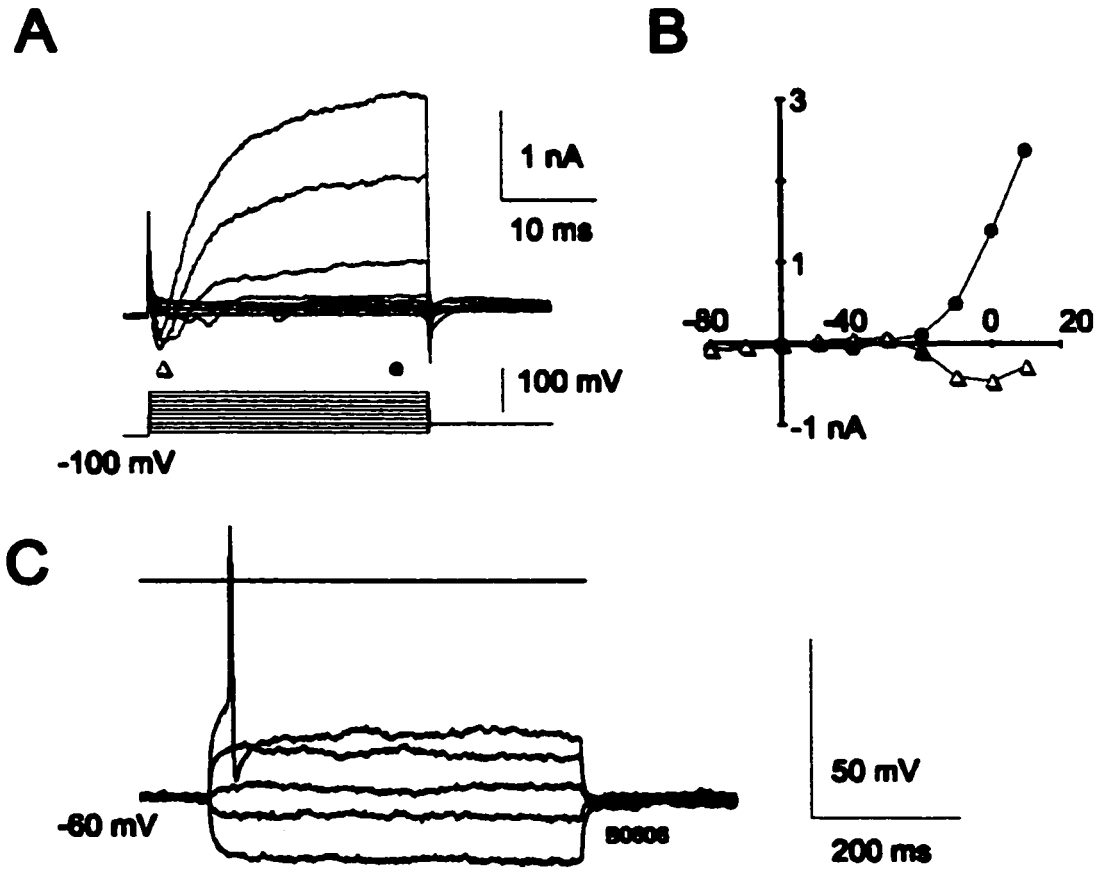

A
1 nA
10 ms
100 mV
-100 mV
B
3
1
-1 nA
-80
-40
0
20
C
-60 mV
50 mV
200 ms

Figure 13. Electrical properties of Dogiel type II enteric neurons. **A.** Current responses (top) to 10 mV voltage steps (bottom) from −100 mV in a typical enteric neuron. Transient inward currents are prominent at the beginning of the responses followed by large slowly developing outward currents. The brief inward deflections with latencies longer than 2-3 ms after the beginning of the voltage step indicate the presence of unclamped voltage-dependent conductance in the processes of these neurons, most probably in the axon. **B.** Current-voltage relationship formed by plotting the amplitudes of the currents in **A** against the corresponding membrane potentials. The open triangles and closed circles represent data from early and late in the response respectively (exact times are indicated by the symbols below the current traces in **A**. **C.** Current-clamp behavior of the same neuron. Current inputs are 30 pA apart. Use of larger current inputs in this and other selected neurons failed to elicit multiple spiking.

not present in our enteric cell cultures (Figure 12). Thus, our cell cultures were composed exclusively of enteric neurons, glia, and small numbers of isolated fibroblasts.

Electrophysiology

The electrical properties of the morphologically-identified Dogiel type II neurons tested were similar to those previously described for neurons of the guinea pig myenteric plexus (Hanani et al., 2000; Tack and Wood, 1992). Figure 13 shows an example of recordings from one such neuron. Figure 13A illustrates the currents evoked in voltage clamp on stepping the membrane potential from -100 mV to +10 mV in increments of 10 mV. The corresponding current-voltage relationships at the beginning and end of the voltage step are plotted in Fig. 13B and reveal that the transient inward component and the more slowly developing outward component visible in Fig 13A both activate near -30 mV. These currents were able to support the generation of a single action potential as shown in Fig. 13C.

Neurochemistry and morphology

Staining for neuronal subtypes, as indicated by neurotransmitter content, demonstrated VIP-, GABA-T-, SOM-, Enk-, and NOS-positive neurons to be present in cultures (Figure 14). Although extensive networks of neuronal processes were positive for Met-Enk, very few neurons (4.4% $\pm$ 2.8% of total neurons) (n=5 experiments in which 1151 total neurons were counted) were Met-Enk positive (Fig. 14A). VIP-positive neurons also extended many branched fibres. These neurons accounted for 4.1% $\pm$ 1.5% of the total neuronal population (n=5 experiments in which 1202 total neurons were counted) (Fig. 14B). In ENS cultures, 3.7% $\pm$ 1.8% of the total neuronal population demonstrated positive staining for the presence of GABA-T (n=5 experiments in which

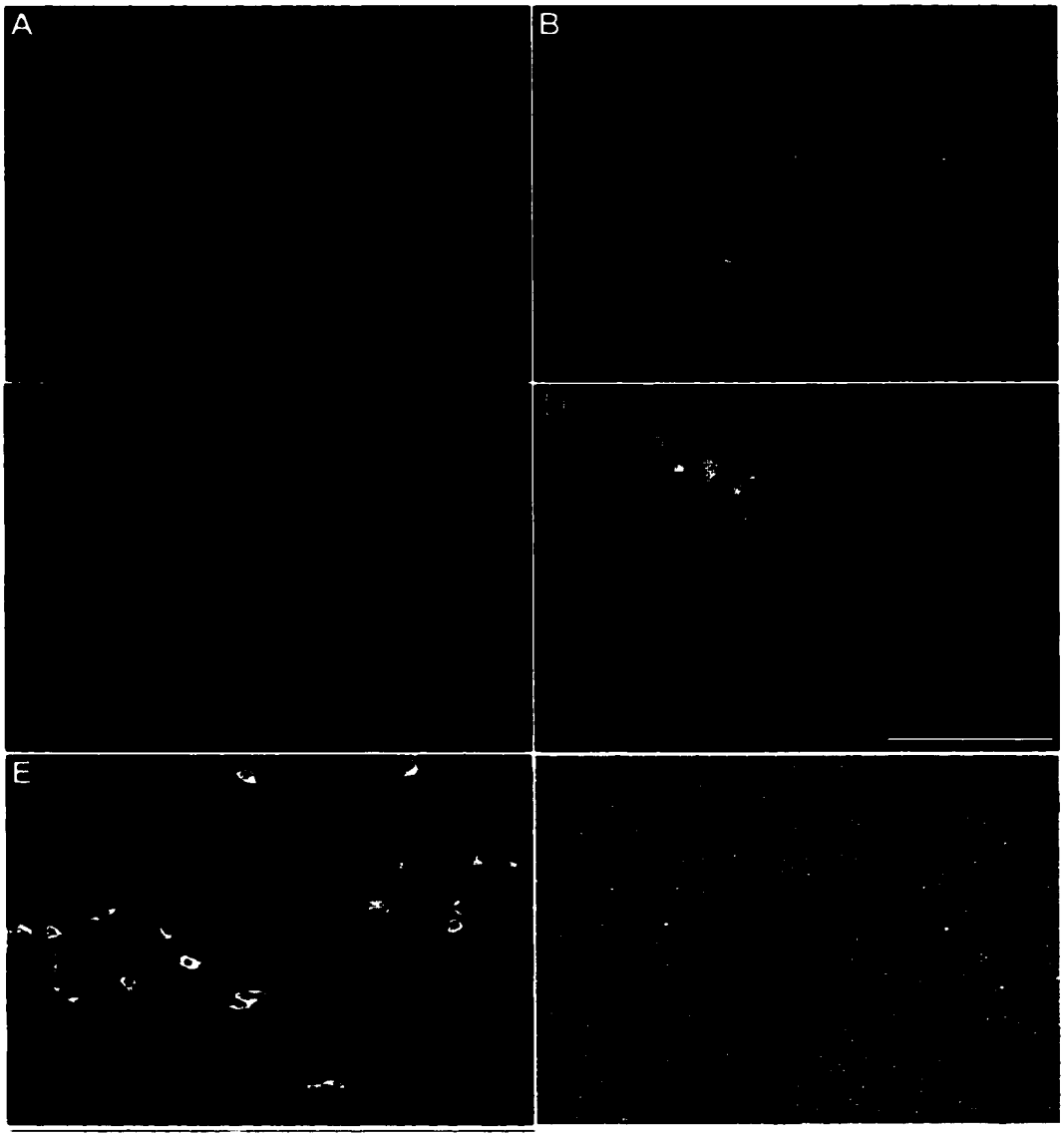

A
B
E

Figure 14. Immunohistochemical and histochemical characterization of cultured neurons. Neuronal subtypes present in 7 day cultures are identified by immunohistochemical staining with anti-met-enkephalin (A), anti-VIP (B), anti-GABA-T (C), anti-somatostatin (D), and anti-NOS (E) antibodies. Histochemical staining with NADPH-diaphorase indicates nitric oxide synthase activity within NOS-positive neurons. (F). Bars represent 100 microns.

1222 total neurons were counted) (Fig. 14C). A similar percentage of neurons (3.7% ± 1.3% of total neurons) (n=5 experiments in which 1182 total neurons were counted) was immunoreactive for somatostatin (Fig. 14D). Large numbers of NOS-positive neurons were observed, which constituted 33.5% ± 7.7% of total neurons (n=5 experiments in which 1162 total neurons were counted) (Fig. 14E). Finally, substance P immunoreactivity was observed in 23.5% ± 6.4% of total neuronal populations (n=5 experiments in which 1204 total neurons were counted).

Morphological characterization of cultured neurons identified Dogiel/Stach types I and II neurons (Dogiel, 1899; Stach, 1989) in culture; those morphological subtypes which constitute the majority of myenteric plexus neurons *in vivo* (Furness and Costa, 1987b) (Figure 15). Identification of the Dogiel/Stach type morphologies of 100 cultured neurons identified 48% Dogiel type I neurons, 36% Dogiel type II neurons, 9% Stach type IV neurons, and 7% neurons of indeterminate morphology.

ENS Responses to Toxic Insult

Within our ENS cultures, neurodegeneration in response to insult was readily detected by several assays. Neuronal loss could be quantitated by comparison of neuronal numbers (identified by anti-NSE staining) within control (Fig. 16A) and experimental (Fig. 16B) cultures. TUNEL staining identified apoptotic cells within cultures (Fig. 16C), while Fluoro-Jade staining marked necrotic cells (Fig. 16D). Apoptosis was localized to neurons by double staining for NSE and Hoechst 33258. Identified neurons (Fig. 16E) were examined for chromatin condensation characteristic of apoptosis (Fig. 16E', Fig. 16E"). Apoptosis was also identified in live cells by annexin-

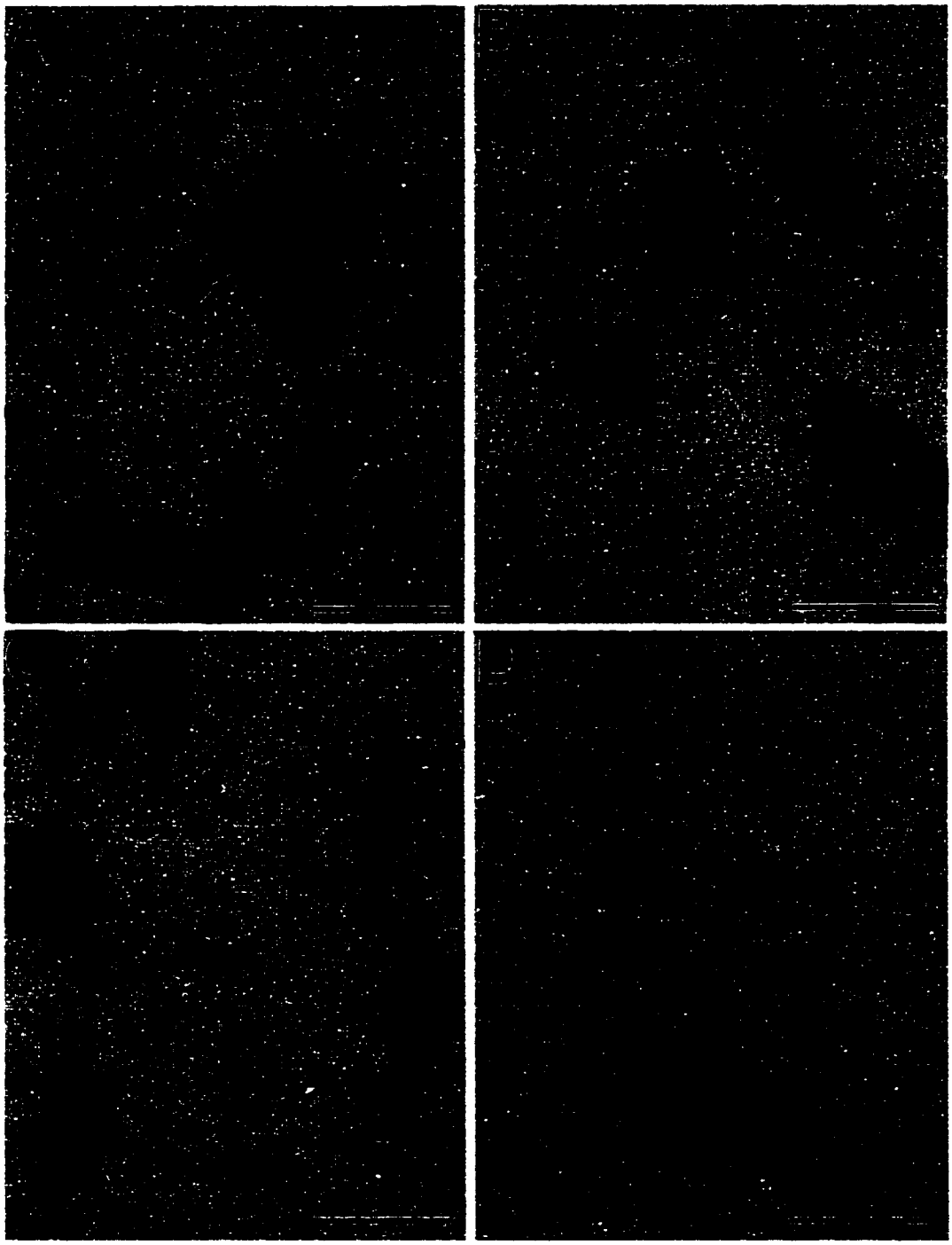


Figure 15. Observation of Dogiel/Stach-type neuronal morphology in cultures. Fixed 7 day cell cultures were histochemically stained with NADPH-diaphorase for morphological classification of enteric neurons. Identified type I cells (A & B), indicated by arrows, are typically flattened neurons with stellate or angular forms, 4-20 short dendrites and a single axon. Identified type II cells (C & D) are characteristically angular, star, or spindle-shaped neurons with 3-10 long, often branching dendrites, and one axon. Bars represent 50 microns.

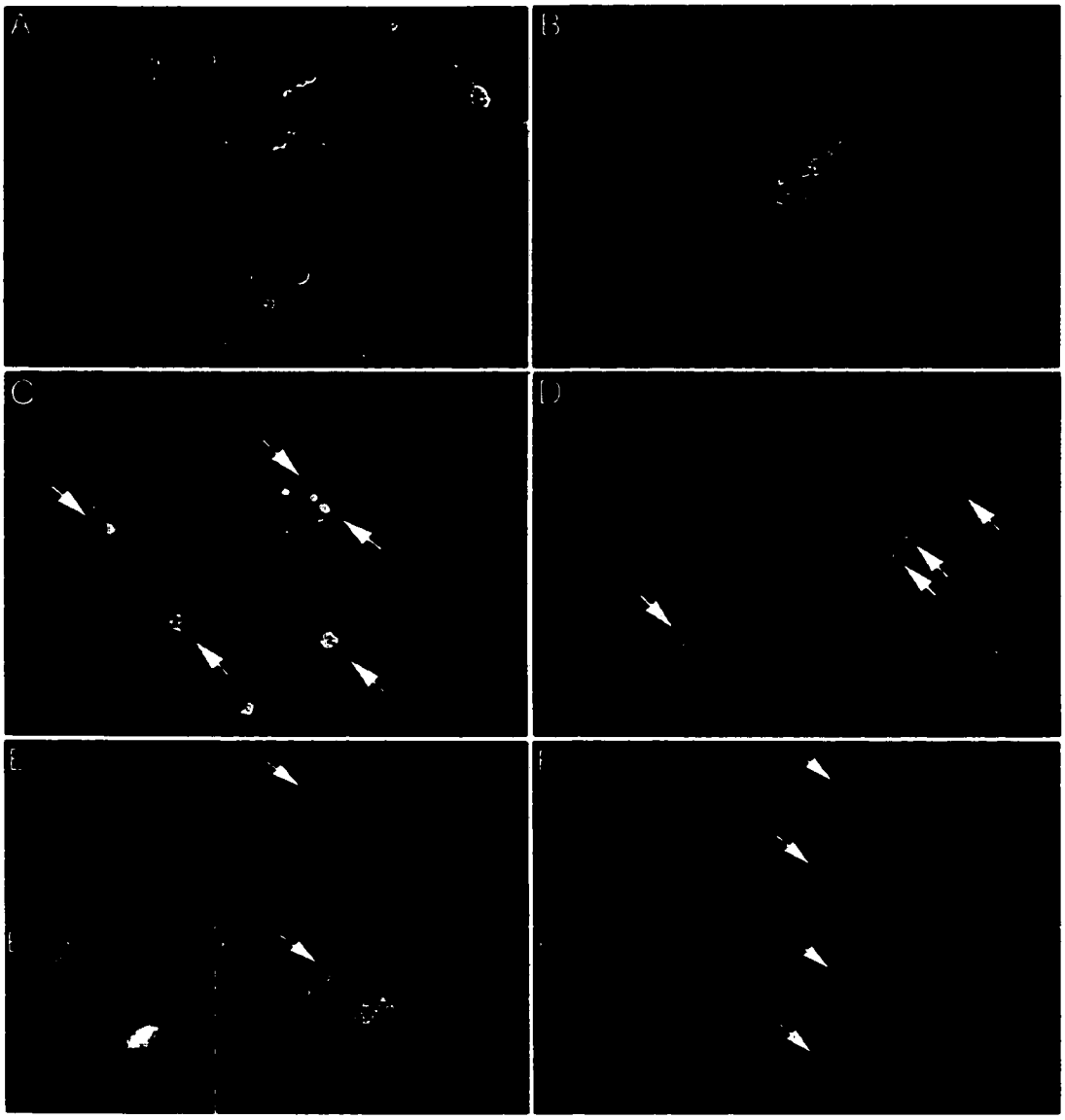

A
B
C
D
E
F

Figure 16. Toxicological characterization within cultures. Identification of neuronal cell death and characterization of cell death mechanisms are demonstrated in cytotoxically challenged cultures. Anti-NSE staining demonstrates the decrease in neuronal numbers from control cell cultures (**A**) to those exposed to 24 hr cytotoxic challenge (**B**). TUNEL staining identifies actively apoptotic cells, as indicated by arrows (**C**). Fluoro-Jade staining identifies necrotic cells, as indicated by arrows (**D**). The NSE-labeled neuron in (**E**) (indicated by arrow), is identified as actively undergoing apoptosis by its characteristic chromatin condensation shown with Hoechst 33258 staining (**E''**). This chromatin condensation is also shown at higher magnification (**E'**). Apoptotic cells are also distinguished from dead or necrotic cells by annexin - propridium iodide staining. Annexin staining (**F**) identifies apoptotic, necrotic, and dead cells indiscriminately. Non-apoptotic cells (ie. necrotic or dead cells), indicated by arrows, are identified by propridium iodide (**F'**). Apoptotic cells are those labeled by annexin but not propidium iodide. Bars represent 100 microns except in E' in which the bar represents 20 microns.

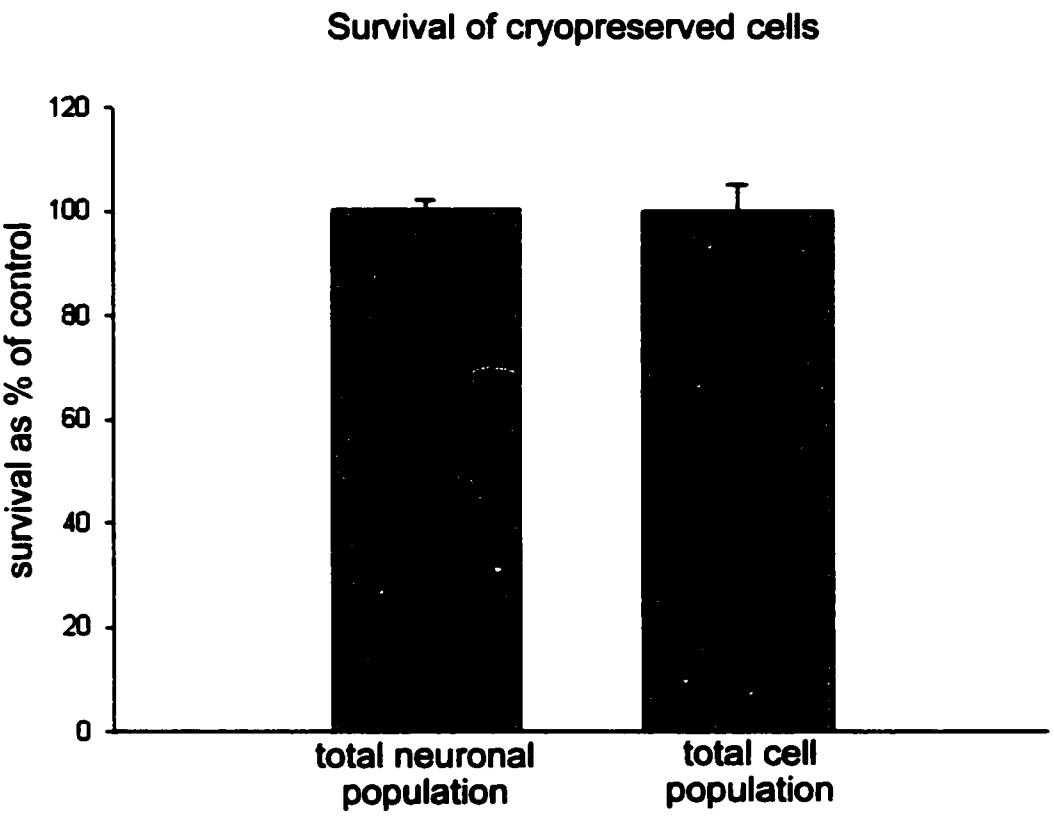

Survival of cryopreserved cells
survival as % of control
120
100
80
60
40
20
0
total neuronal population
total cell population

Figure 17. Viability of cryopreserved enteric neurons. The viability of both total neuronal populations and total cell populations within cryopreserved cultures do not significantly differ from those of freshly plated control cultures isolated from the same animal. Cell survival in cryopreserved cultures is expressed as a percentage of that of the same population in freshly plated controls. In all cases, survival is evaluated in 7 day cell cultures, and the data are the result of n=5 experiments.

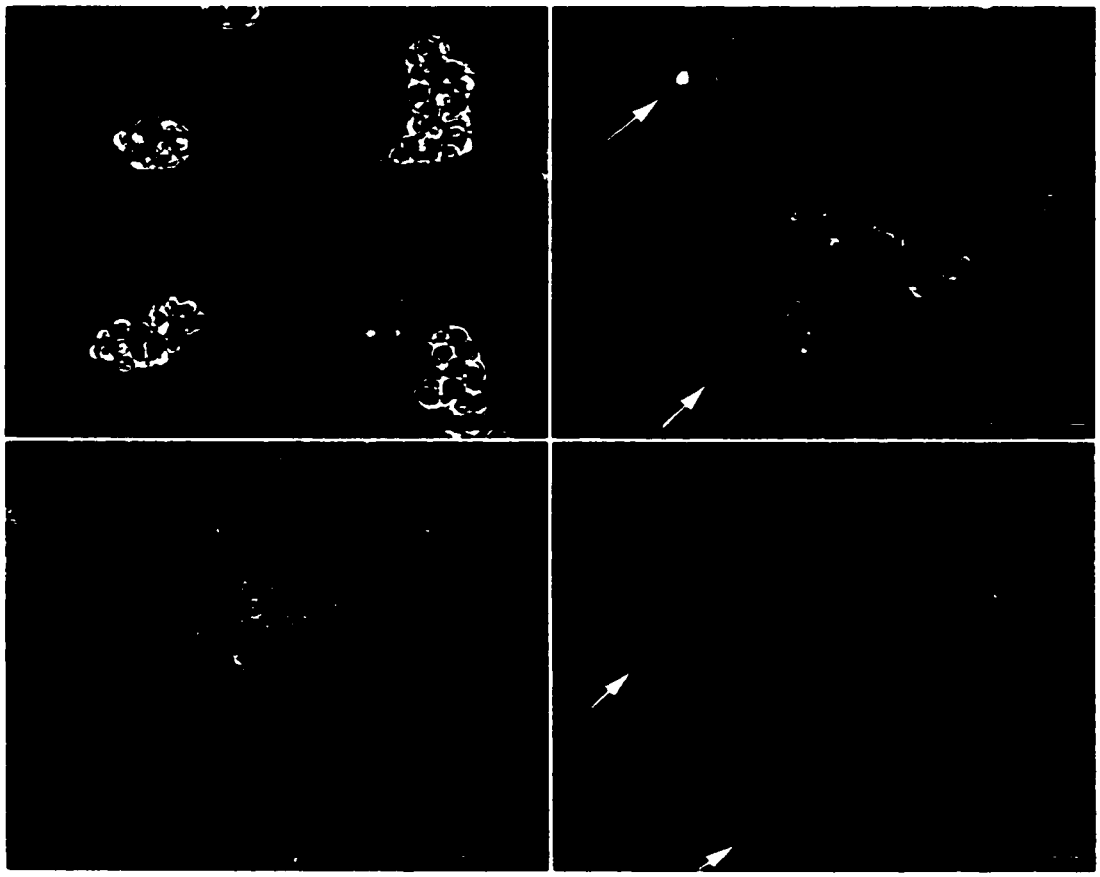


Figure 18. Time course of morphological differentiation of cryopreserved enteric neurons following plating. Live cultures of cryopreserved enteric neurons were characterized over their incubation time course. Upon plating (**A**), all cells exhibited a uniform rounded appearance. After 24 hrs (**B**), cells had attached to the culture substrate and commenced extending processes. By 48 hrs (**C**), cells had flattened onto the culture substrate, extended considerable branching processes, and exhibited characteristic cell-type morphologies. At 7 days (**D**), cellular clusters were extended over the culture substrate, and exhibited a vast network of processes. Bars represent 100 microns.

propridium iodide staining. Cells positively stained for annexin (Fig. 16F) which did not demonstrate propridium iodide staining (Fig. 16F') were identified as actively apoptotic.

Evaluation of Cryopreserved Cell Culture viability

Quantitative evaluation of cell cultures revealed that cell viability for both neuronal populations and total cell populations did not significantly differ between cryopreserved cell cultures and unfrozen controls (Figure 17). The time course of morphological development following plating of cryopreserved cells was characterized over their 7 days of incubation (Figure 18). The appearance of live cells under phase contrast in sister cultures of frozen and control neurons were indistinguishable except that, at 24 hrs following plating, there was noticeably more fiber outgrowth in the cryopreserved neuron cultures (Figure 18B). Whereas, in control cultures at 24 hrs following plating, neuronal aggregates generally extended only a single process, frozen cultures commonly extended multiple processes. Similar results were seen with cells frozen for as long as 3 months. As seen in control cultures, cryopreserved cultures contained glial cells and small numbers of fibroblasts. At 7 days cultures consisted of several neuronal and glial aggregates resembling small myenteric ganglia with vast networks of long, branching processes (Figure 18D).

Immunohistochemical staining identified the same cell types present in both freshly-plated control cultures and cryopreserved cultures. Staining with anti-neuron specific enolase revealed morphologically normal neurons similar to their freshly plated counterparts (Figure 19A). Anti-GFAP staining identified large beds of glial cells (Fig. 19B). Double staining for GFAP and the mesenchymal intermediate filament protein

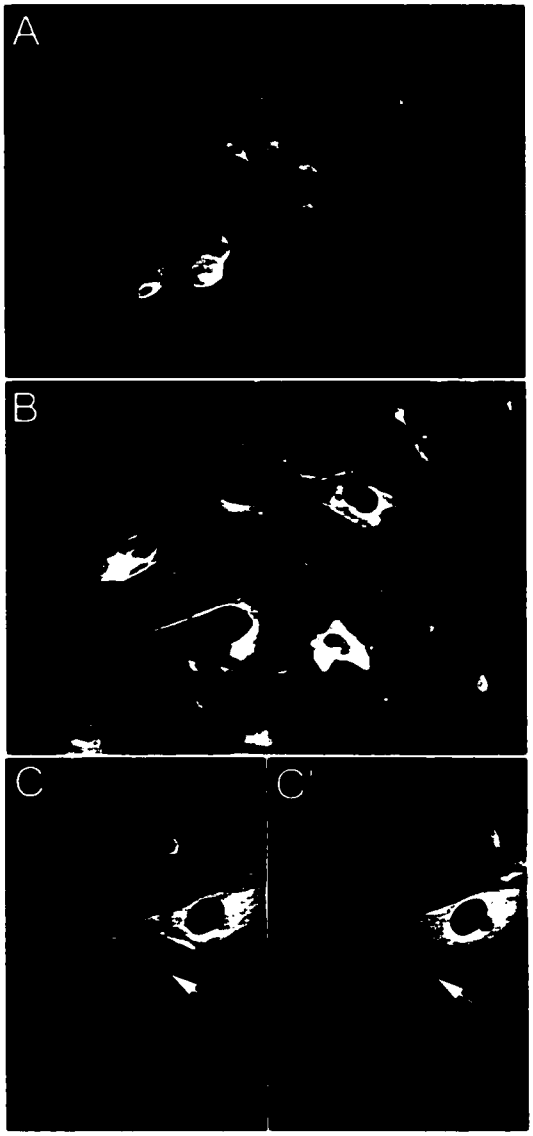

A
B
C
C'

Figure 19. Cell types present in cultures of cryopreserved cells. (**A**) Neurons and their processes are stained for neuron-specific enolase. (**B**) Anti-GFAP antibody staining identifies glial cells in culture. Double staining for GFAP and vimentin distinguishes enteric glia from fibroblasts. Both fibroblasts and glia positively stain for vimentin (**C**), but only glia are immunopositive for GFAP (**C'**). Cells stained for vimentin but not GFAP are fibroblasts (arrows). Bars represent 50 microns.

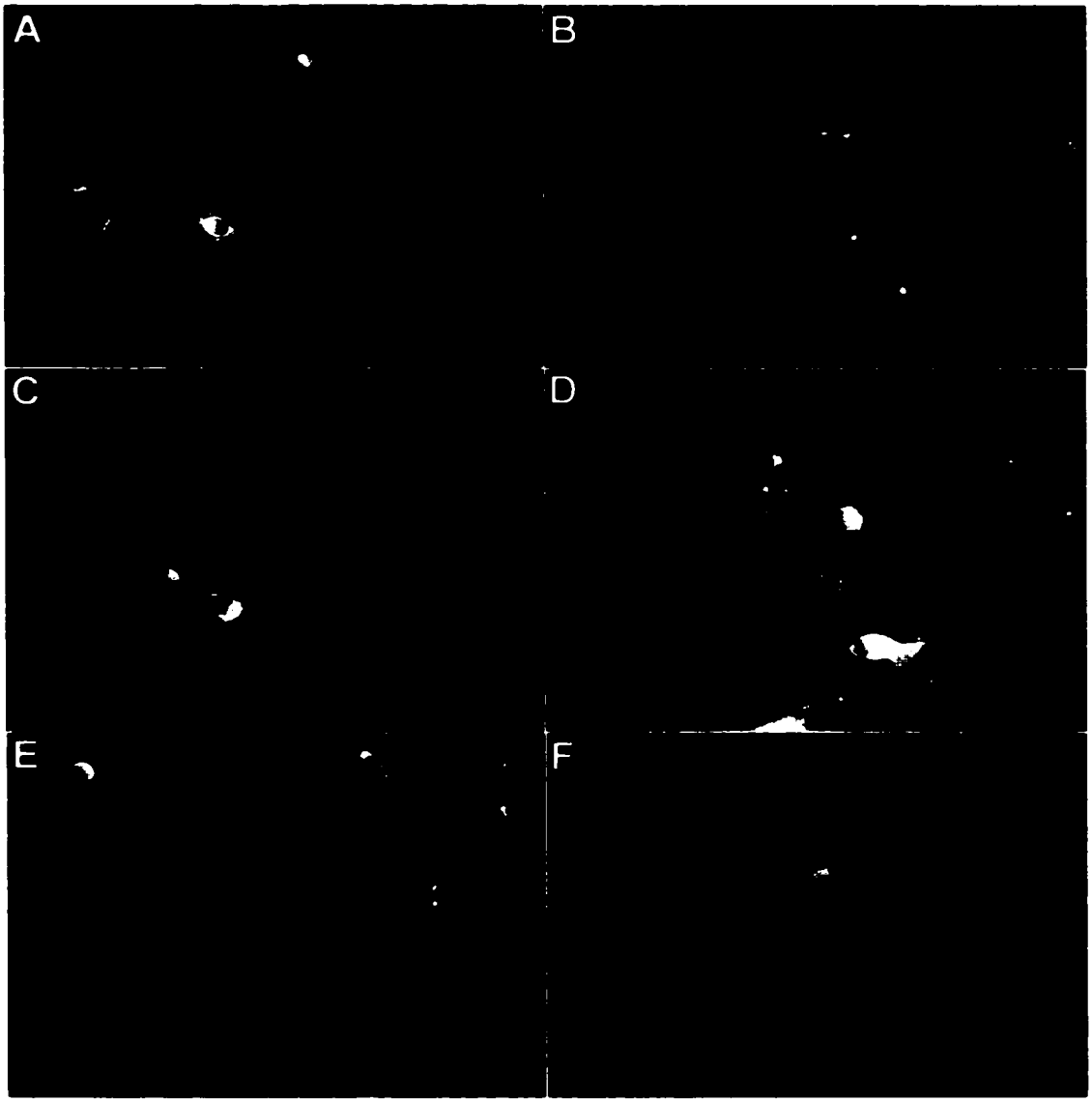

A
B
C
D
E
F

Figure 20. Immunohistochemical characterization of cryopreserved enteric neuronal cultures. Neuronal subtypes present are revealed by immunohistochemical staining with anti-GABA-T (**A**), anti-VIP (**B**), anti-met-enkephalin (**C**), anti-NOS (**D**), anti-substance P (**E**), and anti-somatostatin (**F**). All staining was performed in fixed 7 day cultures. Bars represent 50 microns.

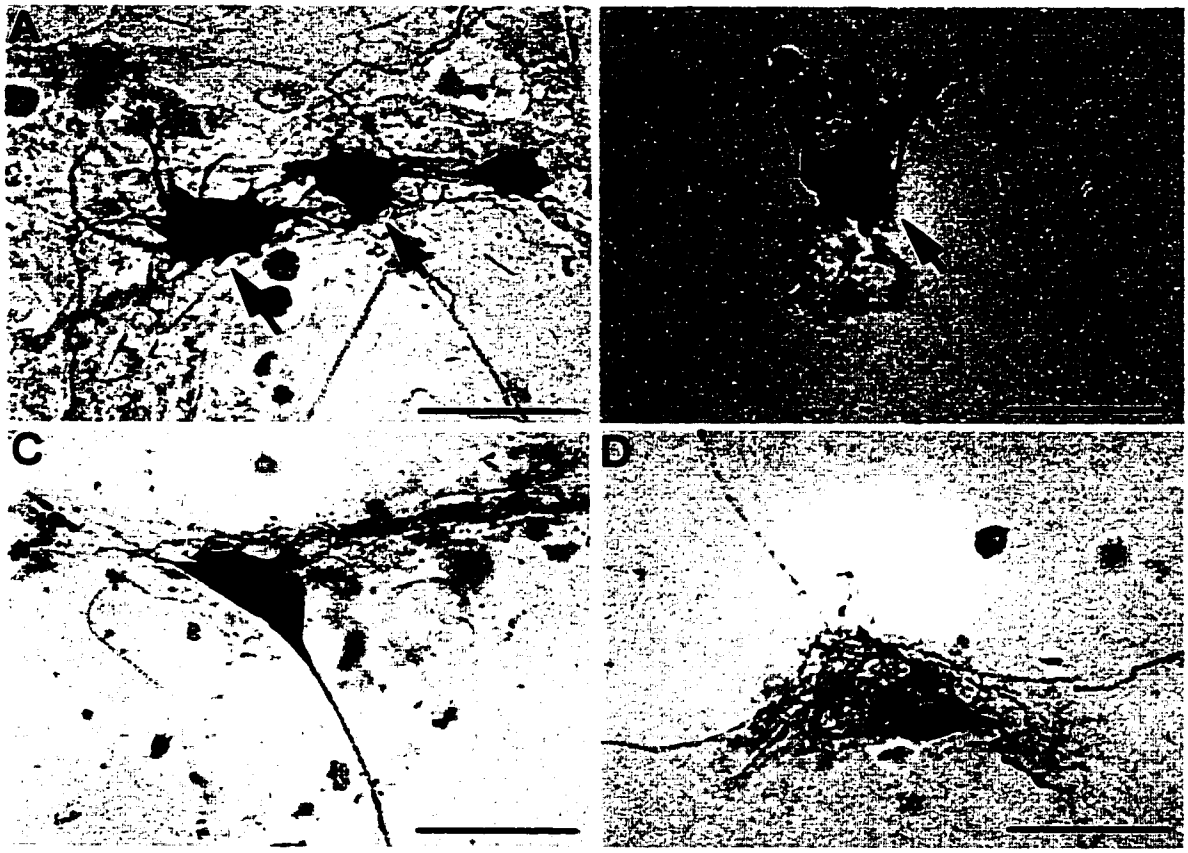

A
C
D

Figure 21. Dogiel/Stach-type neuronal morphology in cultures. Cell cultures were reacted for NADPH-diaphorase to classify enteric neurons morphologically. Type I cells (A & B) are flattened with stellate or angular forms, 4-20 short dendrites, and a single axon. Type II cells (C & D) are angular, star, or spindle-shaped with 3-10 long, branching dendrites and one axon. Bars represent 50 microns.

vimentin distinguished glia from fibroblasts. Whereas large numbers of glia were present, only small numbers of fibroblasts were present in isolated small clusters (Fig. 19C & 19C').

Staining for neuronal subtypes demonstrated that VIP-, GABA-T-, Met-Enk-, SOM-, substance P-, and NOS-positive neurons were present in cryopreserved cell cultures (Figure 20). Quantitation of these neurochemical subtypes of myenteric neurons revealed that 3.4% $\pm$ 1.0% of all neurons are VIP-positive (n=5 experiments in which 1385 total neurons were counted), 2.8% $\pm$1.8% of all neurons are GABA-T-positive (n=5 experiments in which 1300 total neurons were counted), Met-Enk-positive neurons represent 3.3% $\pm$ 1.0% of total neurons (n=5 experiments in which 1471 total neurons were counted), SOM-positive neurons represent 3.1% $\pm$ 0.8% of total neurons (n=5 experiments in which 1282 total neurons were counted), substance P-positive neurons represent 24.5% $\pm$ 8.5% of total neurons (n=5 experiments in which 1404 total neurons were counted), and NOS-positive neurons represent 32.2% $\pm$ 8.5% of total neurons (n=5 experiments in which 1616 total neurons were counted). NOS neuron activity was shown by NADPH-diaphorase staining in cryopreserved cell cultures (Figure 21), and permitted identification of morphological neuron types. Dogiel types I and II neurons and Stach type IV neurons were identified within cultures (Dogiel et al., 1899; Stach, 1989). The relative distributions of these morphological neuronal subtypes were determined within anti-PGP 9.5-stained cultures. Of 100 neurons studied, 53% demonstrated Dogiel type I morphology, 42% were of Dogiel type II morphology, 3% were Stach type IV neurons, and 2% were of indeterminate morphology.

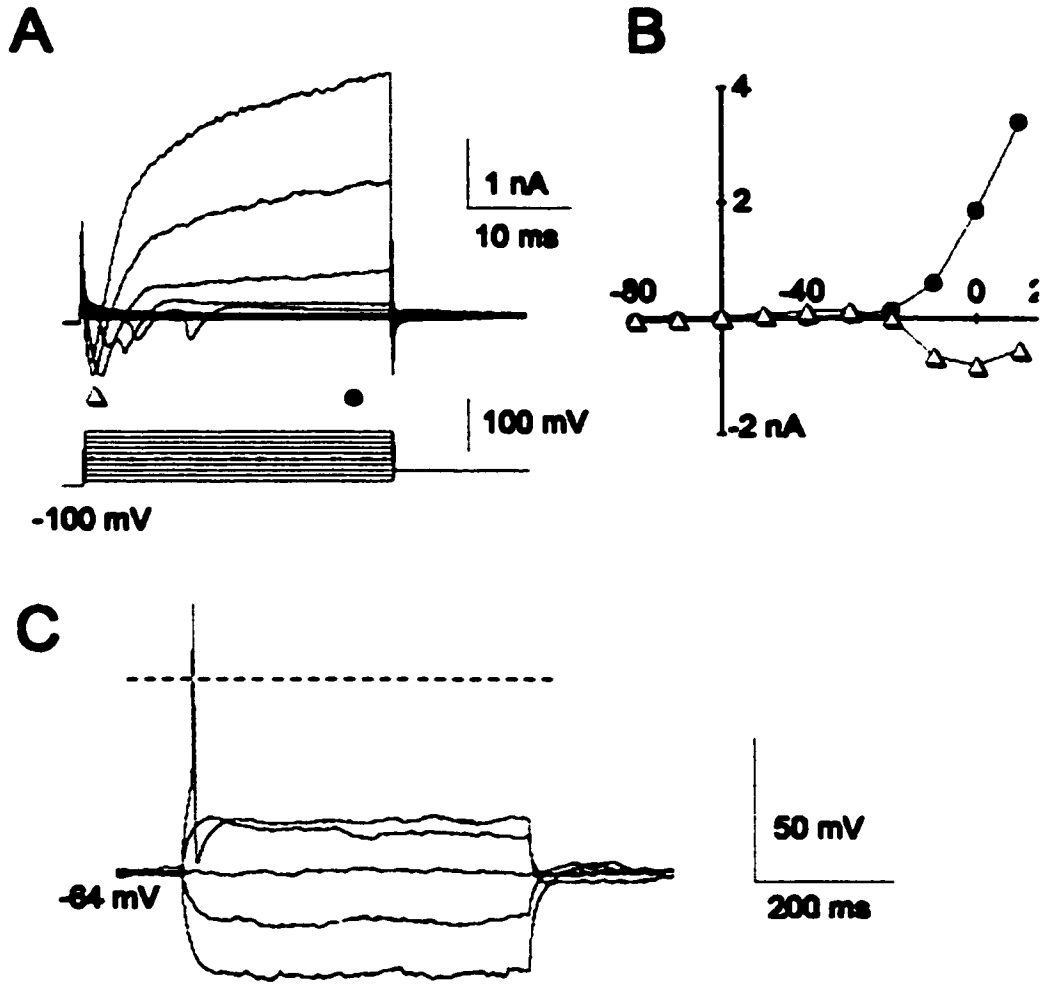

A
1 nA
10 ms
100 mV
-100 mV
B
4
2
-80
-40
0
-2 nA
C
-64 mV
50 mV
200 ms

Figure 22. Electrical properties of Dogiel type II cryopreserved enteric neurons. A. Current responses (top) to 10 mV voltage steps (bottom) from −100 mV in a typical enteric neuron. Transient inward currents are prominent at the beginning of the responses followed by large slowly-developing outward currents. The brief inward deflections with latencies longer than 2-3 ms after the beginning of the voltage step indicate the presence of unclamped voltage-dependent conductances in the processed of these neurons, most probably in the axon. **B.** Current-voltage relationship formed by plotting the amplitudes of the currents in (**A**) against the corresponding membrane potentials. The open triangles and closed circles represent data from early and late in the response respectively (exact times are indicated by the symbols below the current traces in (**A**). **C.** Current-clamp behavior of the same neuron. Dashed line indicates zero millivolts. Current inputs are 30 pA apart. Use of larger current inputs in this and other neurons failed to elicit multiple spiking.

Electrical recordings in the whole-cell patch configuration were performed on n = 25 cryopreserved enteric neurons with gross morphological characteristics of Dogiel type II neurons after 6-9 days *in vitro*. The basic electrical properties of these neurons, including the mean values of the resting membrane potential (-52 ± 2.3 mV), and input resistance (503 ± 71 MΩ) were comparable to those of enteric neurons in acutely isolated whole myenteric ganglia (Hanani et al., 2000). Current-voltage relationships, determined by holding neurons near -100 mV and stepping to more positive voltages, revealed transient inward currents and sustained outward currents at potentials more positive than approximately –30 mV. A typical example is shown in Figs. 22 A, B. The peak amplitudes of the sustained outward (presumably K^+ dependent) currents were considerably larger than those of the transient inward currents (Fig 22B) resulting in a limited ability to generate action potentials. Thus, only 12 of the 23 neurons tested under current clamp conditions were capable of supporting action potentials and multiple spiking was not seen. In those cells that did possess action potentials, however, spikes were well developed with overshooting peaks and prominent fast afterhyperpolarizations (Fig. 22C) consistent with the known properties of enteric neurons (Tack et al, 1992).

General observations in chemical insult experiments

Primary cultures of rat myenteric neurons characteristically consisted of neuronal cell clusters overlaying glial meshworks, and small isolated groups of fibroblasts. Neurons exhibited round or elongate cell bodies and an extensive network of processes extending to neighbouring cells and were easily distinguished from other cells. Under control conditions very few cells (generally less than 1%) were observed undergoing apoptosis (Fig. 23). Following 24 and 48 hrs serum deprivation, total neuronal numbers

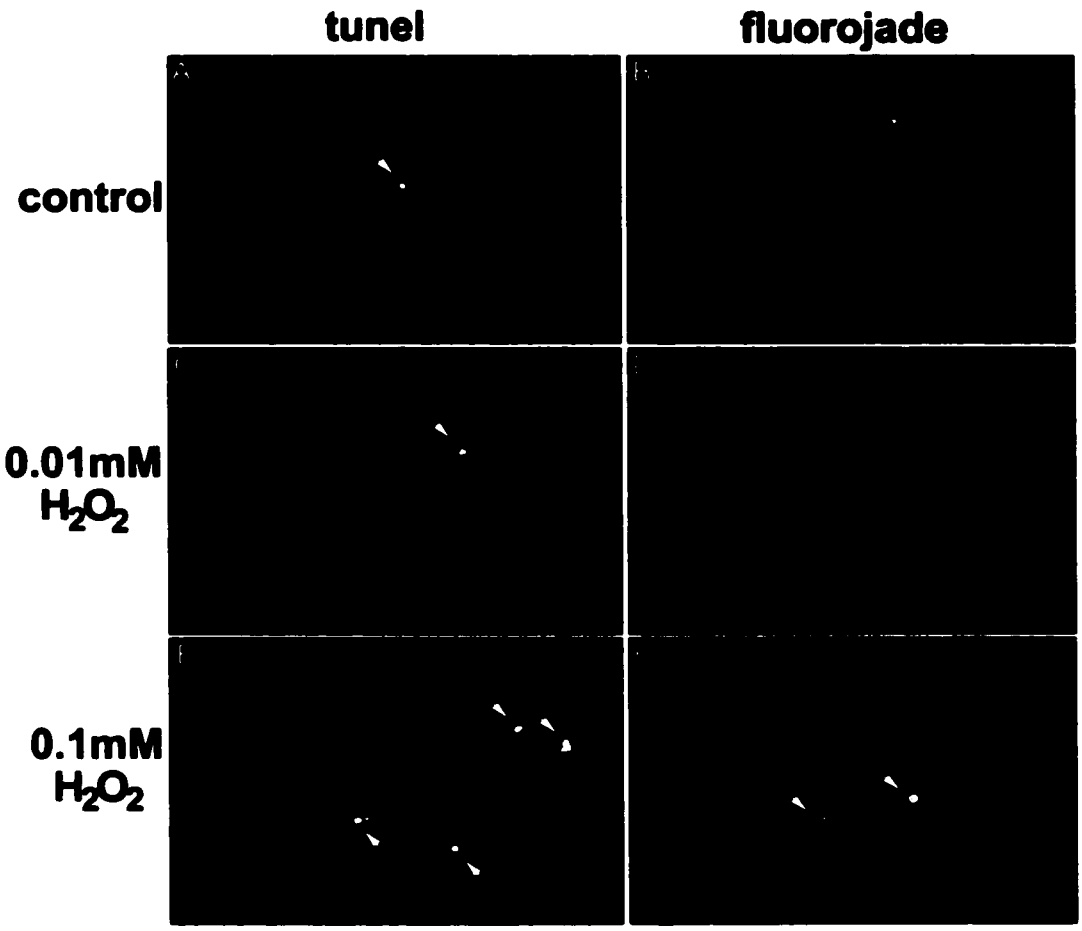

tunel
fluorojade
control
0.01mM
H_2O_2
0.1mM
H_2O_2

Figure 23. Cell death mechanisms involved in concentration dependent H_2O_2 induced cell death. Cultures were exposed for 24 hrs to vehicle (**A**, **B**), 0.01 mM H_2O_2 (**C**, **D**), or 0.1 mM H_2O_2 (**E**, **F**). Panels on the left (**A**, **C**, **E**) have been treated for TUNEL indication of actively-apoptotic cells. Panels on the right (**B**, **D**, **F**) have been treated with Fluoro-Jade to reveal necrotic cells. In this figure, arrows indicate apoptotic and necrotic cells. Apoptotic cell death predominates at lower toxin concentrations than necrosis. Bars indicate 100 microns.

were significantly reduced (67.2% survival ± 8.5%, and 2.5% survival ± 0.4% respectively), while Hoechst 33258 staining showed increased numbers of actively apoptotic cells. All toxins tested (HNE, SNP, H_2O_2) induced apoptosis in cultured enteric neurons. At low concentrations of toxins, cell death occurred almost exclusively via apoptosis. At higher toxin concentrations, the occurrence of necrosis increased. Figure 23 illustrates the predominance of these cell death mechanisms in cultures exposed to a range of H_2O_2 concentrations.

HNE-induced cytotoxicity

Exposure of cultures to HNE for 24 hrs induced concentration-dependant toxicity within neuronal populations and total cell populations (Fig. 24). At the highest concentration of HNE tested (200 μM), the majority of remaining neurons were actively undergoing apoptosis after 24 hrs insult (93.18% ± 1.21%). Following such HNE insult, neurons were clumped together and exhibited shrunken pyknotic cell bodies and retracted cell processes. Neurons demonstrated a significantly higher sensitivity to HNE insult than did other cell types in ENS culture, such that at 50 μM and 100 μM HNE, damage was almost completely restricted to neurons (Figure 24). HNE induced significantly more cell death in neurons than in total cell populations at 100 μM concentrations.

NOS neurons showed preferential survival as compared to total neuronal populations upon 24 hr exposure of cultures to 100 μM HNE (Fig. 25A). These neurons were not unaffected by HNE insult however, as they exhibited a significant down-regulation of NOS activity as indicated by NADPH-diaphorase staining. Cultures were exposed to HNE for longer time intervals to determine if this preferential sparing of NOS neurons remained, indicating a true resistance of NOS neurons to HNE damage. Little

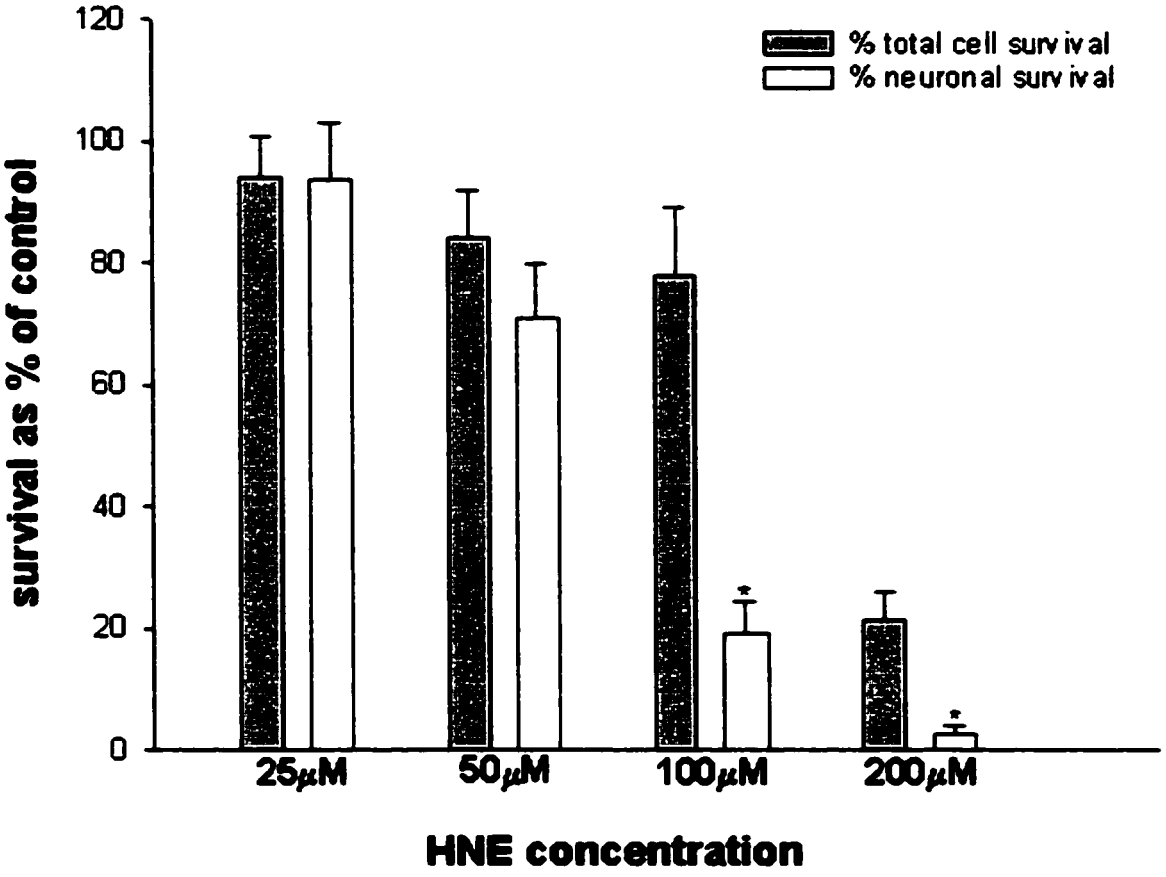
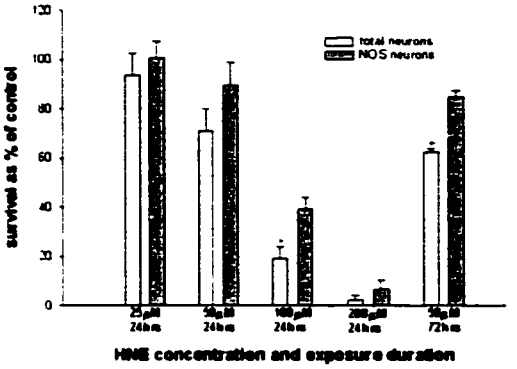
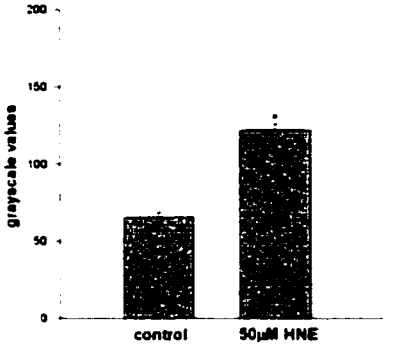

120
100
80
60
40
20
0
survival as % of control
% total cell survival
% neuronal survival
*
*
25μM
50μM
100μM
200μM
HNE concentration

Figure 24. HNE-induced cell death. HNE, applied to 7 day cell cultures for 24 hrs, demonstrates a concentration-dependent toxicity for both neuronal populations and total cell populations. Neuronal cell loss is significantly greater than that of total cell populations following exposure to 100 μM and 200 μM HNE. * $p<0.01$ (one-way ANOVA, Tukey/Kramer analysis).

A


total neurons
NOS neurons
survival as % of control
HNE concentration and exposure duration

B


grayscale values
control
50μM HNE

C

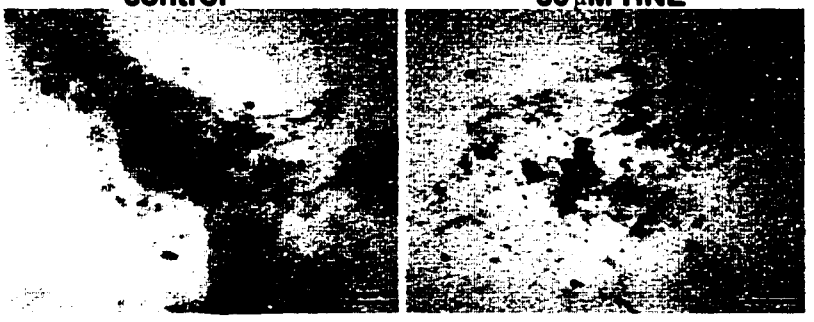

control
50 μM HNE

Figure 25. Preferential sparing of NOS-positive neurons with concomitant down-regulation of NOS activity. **A)** NOS neurons show preferential survival as compared to total neuronal populations upon 24 hr exposure to 100 μM HNE and upon 72 hr exposure of cultures to 50 μM HNE. *$p<0.01$ (two-tailed Student's *t* test). **B)** NOS neurons exposed to 50 μM HNE for 72 hrs exhibit a significant down-regulation of NOS activity compared to controls, indicated by NADPH-diaphorase staining intensity. Increasing grayscale values are indicative of a decreasing staining intensity. For this experiment, n=102 points at which grayscale values were evaluated for each experimental treatment. Grayscale values were taken at three separate points within each neuron evaluated. *$p<0.01$ (two-tailed Student's *t* test). **C)** NADPH-diaphorase staining intensity, indicative of NOS activity, is visibly reduced in HNE-treated cultures as compared to controls. Bars indicate 100 microns.

further cell loss occurred during the additional 48 hr exposure to 50 μM HNE, and, at the 72 hr time point, little active apoptosis was observed. These observations indicate that the majority of apoptosis occurred during the first 24 hr exposure to HNE. Preferential sparing of NOS-positive neurons remained after 72 hr HNE exposure (Fig. 25A), although the intensity of NADPH-diaphorase staining indicated a significant down-regulation of NOS activity in these cells (Fig. 25B, 25C).

SNP toxicity

Exposure of 7 day-old enteric neuronal cultures to varying concentrations (0.1 mM, 0.5 mM, 1 mM) of sodium nitroprusside (SNP) for 24 hrs was observed to induce neuronal cell death in a concentration-dependent manner (Fig. 26A). For all concentrations of SNP tested, neurons exhibited far greater susceptibility to SNP insult than did fibroblasts and glial cells. Typically, SNP exposure caused a pronounced retraction of processes, pyknosis of cell bodies, and clumping of neurons.

Hydrogen peroxide toxicity

Within 24 hrs of application to the feeding medium of 7 day-old enteric neuronal cultures, H_2O_2 induced neuronal cell death. This neurotoxicity was concentration-dependent, with minimal neuronal death induced at 1μM concentrations, whilst virtually all neurons died at 1 mM concentrations (Figure 26B). While neurons were preferentially damaged at 10 and 100 μM concentrations, all cell types present were susceptible to toxic insult in the presence of 1 mM H_2O_2. At all concentrations of H_2O_2 tested, apoptosis was the predominant mechanism of neuronal cell death (see Fig. 23).

Menadione toxicity

Exposure of neuronal cell cultures to menadione, an endogenous generator of

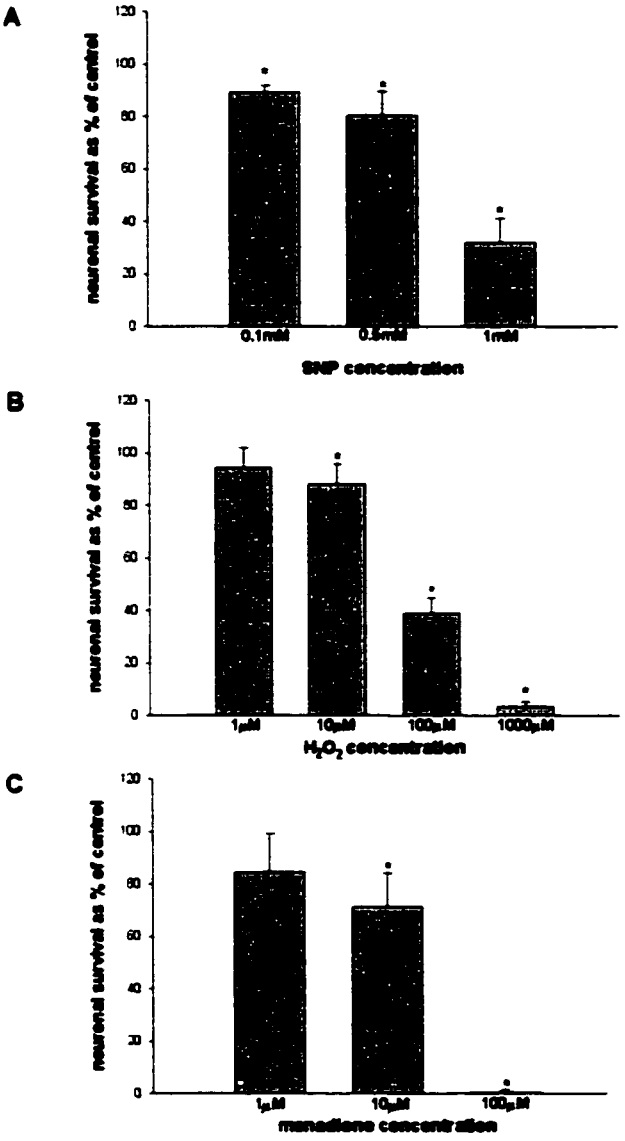

A
neuronal survival as % of control
0
20
40
60
80
100
120
0.1mM
0.5mM
1mM
SNP concentration
B
neuronal survival as % of control
0
20
40
60
80
100
120
1μM
10μM
100μM
1000μM
H2O2 concentration
C
neuronal survival as % of control
0
20
40
60
80
100
120
1μM
10μM
100μM
menadione concentration

Figure 26. SNP, H_2O_2 and menadione demonstrate concentration-dependent toxicities in cultured enteric neurons. **A**) 24 hr exposure of enteric neuronal cultures to concentrations of 0.1 mM to 1 mM SNP induced concentration-dependent neuronal cell loss. **B**) Significant neuronal loss occurred in cultures exposed to concentrations of 10 μM, 100 μM, and 1 mM H_2O_2, which induced concentration-dependent toxicity in these cultures. In **C**), concentrations of 10 μM-100 μM menadione induced significant neuronal cell death in cultures in a concentration-dependent manner. In all cases, *$p<0.01$ (one-way ANOVA, Tukey/Kramer analysis).

H_2O_2, induced cell death in a concentration-dependent manner (Fig. 26C). 10 μM and100 μM menadione induced markedly more neuronal cell death than did the same concentrations of H_2O_2. Widespread death was observed in all cell types with exposure to 100 μM menadione concentrations.

Combined H_2O_2 and SNP Exposure

Combined application of H_2O_2 and SNP for 24 hrs to enteric cultures induced neuronal cell death at very low toxin concentrations (Fig. 27). Exposure to 0.5 mM SNP or 10 μM H_2O_2 individually induced significant, though small amounts of neuronal cell death, while 1 μM H_2O_2 did not itself induce any significant neurotoxicity in cultures. In combination, however, these otherwise low-toxicity doses were highly toxic to cultured neurons. Combination of 1 μM or 10 μM H_2O_2 with 0.5 mM SNP induced significantly more neurodegeneration than the sum of neurodegeneration induced by these compounds individually.

Neuroprotective effects of GDNF

GDNF (10 ng/ml) application to 7 day enteric cell cultures did not itself have any pronounced effects upon neuronal survival (Fig. 28). 24 hr pretreatment of enteric cultures with GDNF was assayed to determine its neuroprotective ability against anoxia-reoxygenation insult, H_2O_2 insult, SNP insult, and combined insult of H_2O_2 + SNP at equieffective concentrations (those which induced approximately 50% neuronal cell death after 24 hr exposure). GDNF pretreatment did not significantly attenuate the toxicity of anoxia-reoxygenation insult, SNP insult, or combined H_2O_2 + SNP insult. Indeed, for 1 hr anoxia-reoxygenation insult that induced the loss of 48.12% ± 9.83% of enteric neurons, 47.3% ± 6.9% neuronal loss occurred with GDNF pretreatment.

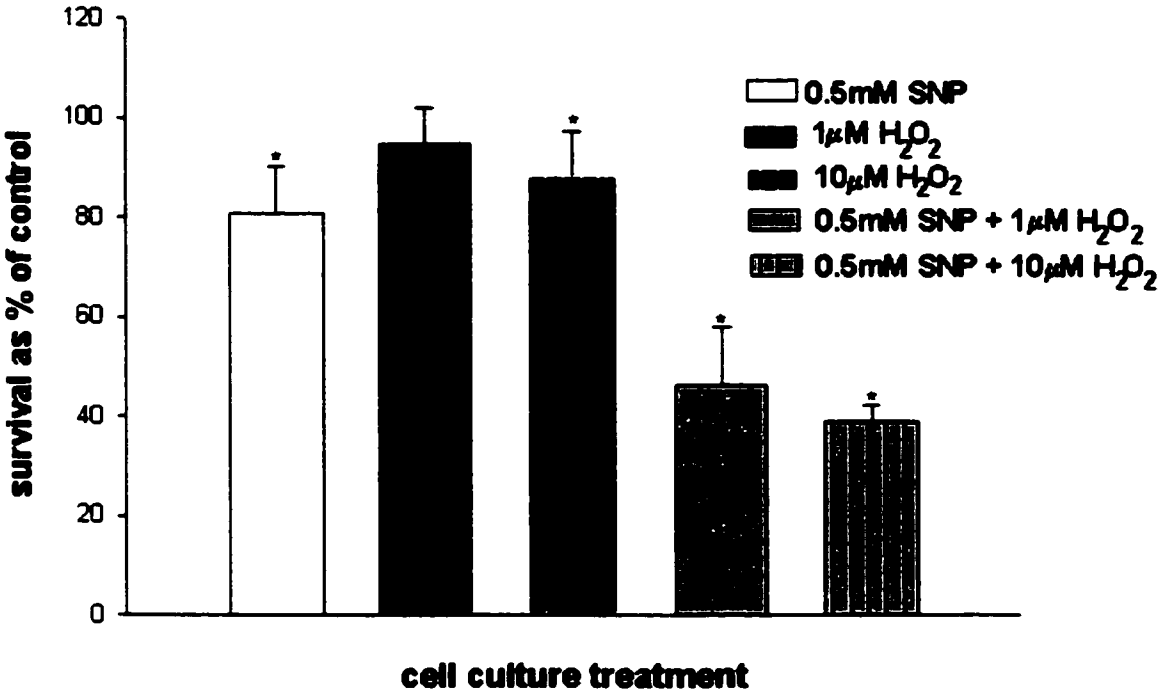

120
100
80
60
40
20
0
survival as % of control
0.5mM SNP
1μM H2O2
10μM H2O2
0.5mM SNP + 1μM H2O2
0.5mM SNP + 10μM H2O2
*
*
*
*
cell culture treatment

Figure 27. H_2O_2 potentiates the toxic properties of SNP on cultured enteric neurons. Combinations of 0.5 mM SNP + 1 μM H_2O_2 and 0.5 mM SNP + 10 μM H_2O_2 induced significantly more neuronal cell death than would result given an additive toxicity of the individual toxins. Significant neuronal cell death was induced by 0.5 mM SNP, 10 μM H_2O_2, 0.5 mM SNP + 1 μM H_2O_2 and 0.5 mM SNP + 10 μM H_2O_2. *$p<0.01$ (one-way ANOVA, Tukey/Kramer analysis).

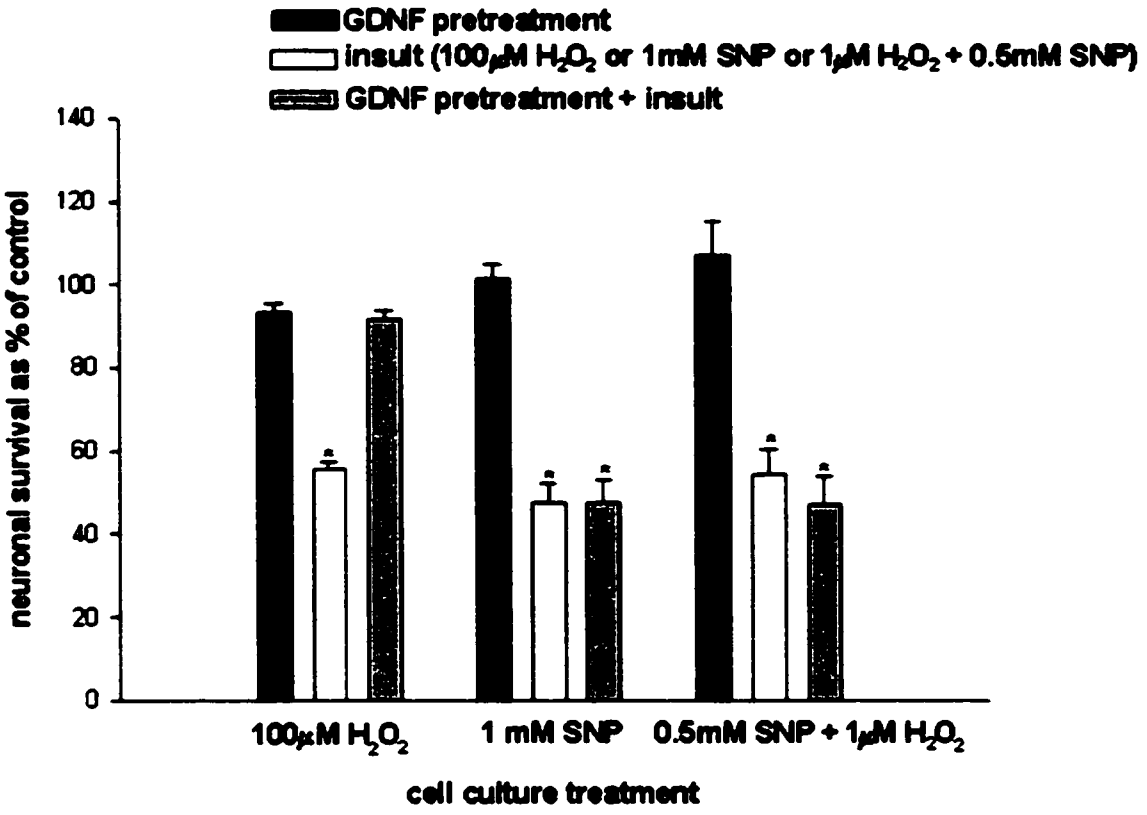

GDNF pretreatment
insult (100μM H2O2 or 1mM SNP or 1μM H2O2 + 0.5mM SNP)
GDNF pretreatment + insult
neuronal survival as % of control
140
120
100
80
60
40
20
0
*
*
*
*
*
*
100μM H2O2
1 mM SNP
0.5mM SNP + 1μM H2O2
cell culture treatment

Figure 28. Ability of GDNF to protect neurons against specific neurotoxic insults. 100 μM H_2O_2 exposure for 24 hrs induced cell death in approximately 50% of cultured ENS neurons. 24 hr pretreatment of ENS cultures with 10ng/ml GDNF prior to such insult effectively prevented this H_2O_2 –induced neurodegeneration. In contrast, GDNF pretreatment did not significantly alter the neurotoxicity induced by either 1 mM SNP or 0.5 mM SNP + 1 μM H_2O_2. *$p<0.01$ (one-way ANOVA, Tukey/Kramer analysis).

However, GDNF pretreatment effectively prevented H_2O_2-induced neuronal cell death.

Cortical culture responses to hypoxic/anoxic insults

Cultured cortical neurons, whose susceptibility to hypoxia-reoxygenation and anoxia-reoxygenation injury has been previously characterized (Goldberg et al., 1987), were employed as positive controls in hypoxia/anoxia experiments. In previous experiments using murine cortical cultures, the vast majority of neurons (80-100%) degenerated following 6 hrs or more of anoxia, whereas little change in neuronal cultures was observed with anoxic exposure of less than 6 hrs duration (Goldberg et al., 1987). Similarly, in our rat cortical cultures, cell death ranged from 32.13% $\pm$ 16.79% of total neurons following 1 hr anoxia, to 56.58% $\pm$ 11.96% of total neurons following 3 hrs anoxia, to 92.46% $\pm$ 5.73% of total neurons degenerating following 6 hrs anoxia. The similarity of the neuronal death responses to anoxia-reoxygenation insult in our cortical neuron cultures to those published previously lends credence to our experimental protocol, and permits some comparison between CNS and ENS susceptibility to such insults.

ENS culture responses to hypoxic/anoxic insults

The effects of hypoxia-reoxygenation and anoxia-reoxygenation insult on cell survival in ENS cultures are shown in Figures 29A and 29B. Whereas complete oxygen deprivation was cytotoxic for both neurons and non-neuronal cells, no significant cell death occurred in response to the oxygen deficiency of hypoxia-reoxygenation. In cultures exposed to anoxia-reoxygenation insult, significant neuronal loss occurred under all experimental conditions tested. Thus, for cultured enteric neurons, significant neuronal loss occurs following even only 1 hr of anoxia, as determined 23 hrs following

initiation of reoxygenation. Almost complete neuronal loss occurs upon exposure to 6 hrs anoxia followed by 18 hrs reoxygenation, or following 24 hrs anoxia alone. In these experiments, increases in neuronal cell death corresponded with increases in anoxic exposure.

Both neuronal and non-neuronal survival were impaired by anoxia-reoxygenation insult in these 24 hr experiments (see Figures 29A and 29B). However, non-neuronal cells showed far less sensitivity to anoxia-reoxygenation-induced cytotoxicity than did enteric neurons. Significant loss of non-neuronal cells occurred only following 6 hr anoxia – 18 hr reoxygenation and 24 hr anoxic insults, whereas significant neuronal cell loss was observed following all anoxia-reoxygenation insults tested.

Elucidation of anoxia and reoxygenation processes in ENS degeneration

In further experiments, the roles of anoxia and reoxygenation in neuronal cell death were examined. Anoxia alone was observed to induce enteric neurodegeneration. As the duration of anoxic insult was increased, neurodegeneration increased in ENS cultures (Figure 30). Reoxygenation following anoxic exposure also contributed to the neurodegeneration of ENS cultures (Figure 30). For both of the insult durations examined, both anoxia and anoxia-reoxygenation insults induced significant neuronal cell loss; however, significantly more neuronal cell loss occurred in response to anoxia-reoxygenation insult than to anoxic insult of the same total duration. 3 hrs anoxia induced approximately 20% enteric neuronal cell loss in cultures. Subsequent reoxygenation of cultures for 2 hrs induced a further approximately 60% neuronal cell loss, as compared to mere additional 30% neuronal cell loss when anoxia was continued for the same 2 hr period (Figure 30).

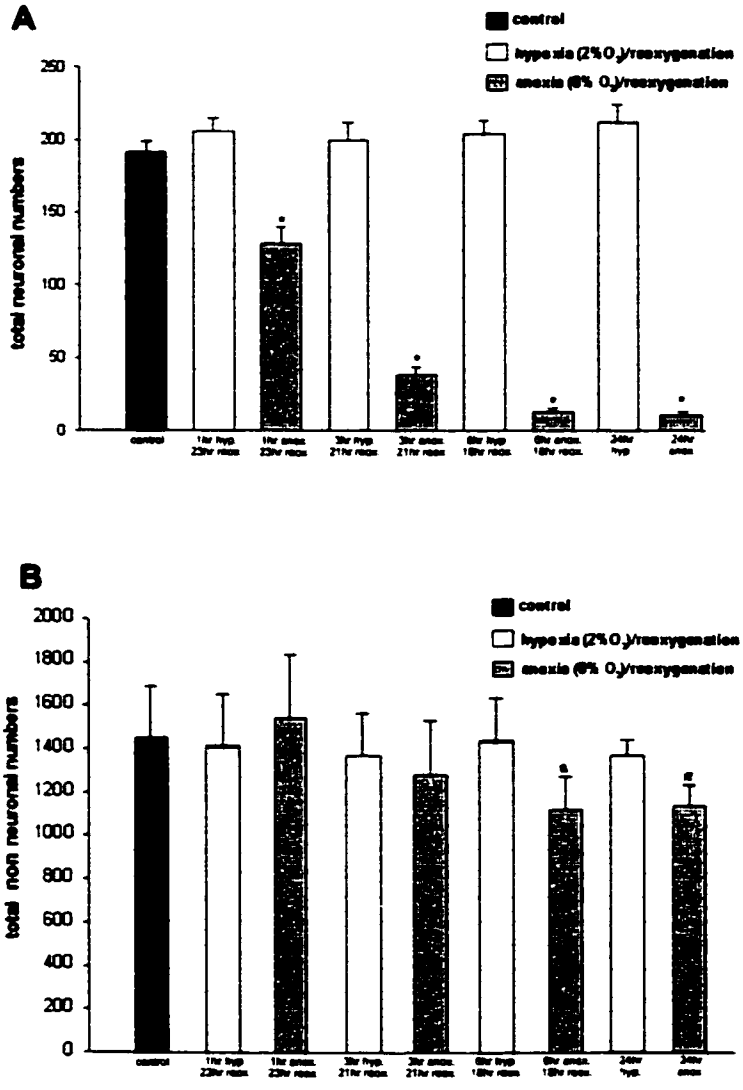

A
control
hypoxia (2% O2)/reoxygenation
anoxia (0% O2)/reoxygenation
total neuronal numbers
250
200
150
100
50
0
B
control
hypoxia (2% O2)/reoxygenation
anoxia (0% O2)/reoxygenation
total non neuronal numbers
2000
1800
1600
1400
1200
1000
800
600
400
200
0

Figure 29. Survival of cultured enteric neurons and non-neuronal cells exposed to hypoxia-reoxygenation and anoxia-reoxygenation insults. No significant cell loss occurred as a result of hypoxia-reoxygenation (O_2 = 2%) for any of the experimental insults tested. **A.** Significant neuronal cell loss was observed at all anoxia-reoxygenation insults tested (O_2 <0.01%). Increases in neuronal cell loss correspond with increases in anoxic exposure duration. **B.** In contrast to neurons, non-neuronal cells in culture (glia and fibroblasts) were highly resistant to anoxia-reoxygenation insult. Significant cell loss occurred only in response to 6 hr anoxia-18 hr reoxygenation and 24 hr anoxia insults. *p<0.01 (one-way ANOVA followed by post-hoc Tukey/Kramer analysis).

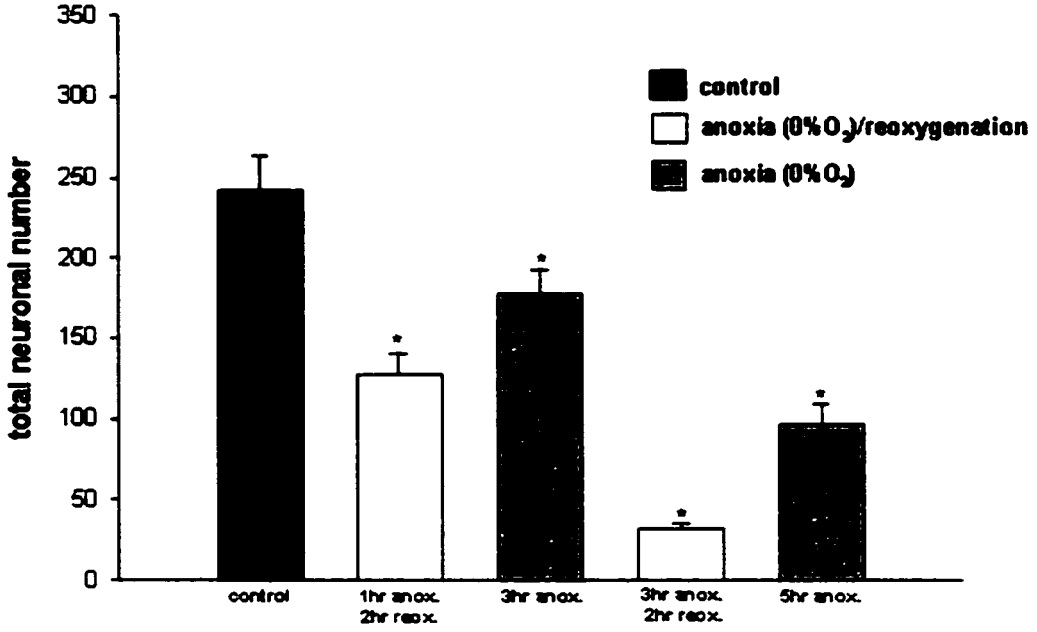

total neuronal number
350
300
250
200
150
100
50
0
control
anoxia (0% O2)/reoxygenation
anoxia (0% O2)
control
1hr anox. 2hr reox.
3hr anox.
3hr anox. 2hr reox.
5hr anox.
*
*
*
*

Figure 30. Comparison of the toxicity of anoxia and anoxia-reoxygenation insults on enteric neuronal cultures. Anoxia induced enteric neurodegeneration in our ENS cultures. Significant neuronal death also occurred during the reoxygenation period following anoxic insult (3 hrs anoxia vs. 3 hrs anoxia followed by 2 hrs reoxygenation). Substantially more neuronal cell death occurred in response to anoxia-reoxygenation insult than to anoxic insult for equivalent time periods (3 hrs and 5 hrs total insult) - indicating the importance of reoxygenation in neuronal injury. These results demonstrate that, for this time course of injury, reoxygenation demonstrated greater toxicity for cultured enteric neurons than did an equivalent time period of anoxia. *$p<0.01$ (one-way ANOVA followed by post-hoc Tukey/Kramer analysis).

Discussion

We report here the successful development of a protocol for culturing dissociated enteric neurons from the myenteric plexus of the adult rat ileum and the suitability of these cultures for electrophysiological and neurotoxicological experimentation. This protocol incorporates an enzymatic digestion followed by mechanical dispersion similar to the method first developed for neonatal myenteric plexus culture by Nishi and Willard (1985). Similar to these neonatal cultures, our enteric cell cultures from adult animals were composed exclusively of neurons, glial cells, and occasional isolated fibroblasts. The high quality of neurons in our cell cultures may in part be due to the presence of glia since it has been reported that neuron-glial cell co-culture improves neuronal survival over that seen during the selective culture of neurons (Saffrey et al., 1991).

Within the dissection, dissociation, and culture processes, several steps are intrinsic to the success of these ENS cultures of myenteric neurons from the adult rat ileum. An initial rapid isolation of ileal tissue from the sacrificed animal is required to minimize hypoxic damage to this tissue. The continuous oxygenation of tissue prior to, during, and following dissection also minimizes hypoxic damage of cells. Adult tissue dissection procedures differ from embryonic and neonatal tissue dissection procedures, in which it is seldom necessary to oxygenate tissue preparation solutions due to the largely anaerobic metabolism of these tissues (Higgins and Banker, 1998). In contrast, incubation of tissues in 4°C Kreb's solution is a common procedure in both neonatal and adult ENS culture protocols, and is employed for maintenance of tissue viability and for minimization of degenerative changes (Hanani et al., 2000; Kirchgessner et al., 1997).

Isolation of the myenteric plexus is a far more difficult procedure in the adult animal than in fetal or neonatal animals due to the presence of fully developed connective tissue sheaths and intestinal tissue layers. Dissection to remove surrounding intestinal tissue layers is commonly performed for the culture of ENS neurons (Kirchgessner et al., 1997; Nishi and Willard, 1985; Hanani et al., 2000). Although this is a time-consuming process, it is considered necessary both to prevent contamination of cultures with unwanted cell types and to minimize the amount of harsh enzymatic digestion required to dissociate tissue. This dissection procedure permits precise recognition and clean dissection of the region of interest, key to successful culture of neurons (Higgins and Banker, 1998).

In all cell culture protocols, it is necessary to maintain tissue and cells in osmotically balanced solutions at physiological pH for all stages of dissection, dissociation and culture (Higgins and Banker, 1998). In our protocol, Kreb's solution was used during tissue dissection, as is standard (Hanani et al., 2000; Kirchgessner et al., 1997). During dissociation, cells were maintained in Hank's balanced salt solution (HBSS) and a Leibovitz's L-15 medium based solution. These solutions are ideal for maintaining cell viability during dissociation procedures, as they provide pH buffering capability in an ambient atmosphere (Higgins and Banker, 1998).

Our dissociation procedure was designed so as to permit efficient dissociation of tissue with minimal damage to the cell types of interest. To that end, tissue was initially digested in a papain-L-cysteine solution. Papain is a purified protease that is commonly accepted to be less disruptive to cells than some other proteases such as trypsin, which can destroy some classes of transmitter receptors on neurons (Segal et al., 1998; Allen et

al., 1988). L-cysteine, a mild reducing agent, is included for its ability to fully activate papain (Hatten et al., 1998). Following inactivation of papain by washes in serum-containing medium, further tissue dissociation is effected by combined collagenase and dispase treatment. Dispase, like papain, is a purified protease used when careful control of the dissociation procedure is required. Collagenase is commonly used for the dissociation of peripheral ganglia from older animals due to the significant amounts of connective tissue present (Johnson and Argiro, 1983). Final mechanical dissociation was accomplished by trituration, which mechanically shears tissue. Previous enzymatic breakdown of cell association components facilitates the mechanical dispersion of cells, minimizing the possibility of cell damage by this process (Higgins and Banker, 1998).

Cell cultures were maintained in a MEM-based medium supplemented with 2.5% rat serum, 2mM glutamine, 15mM glucose, 100U penicillin-streptomycin, 10μM fluorodeoxy-uridine, 1mM cytosine arabinoside and 0.5mM uridine triphosphate. This use of MEM-based media is standard for rat ENS cultures (Nishi and Willard, 1985), while penicillin-streptomycin is commonly added to prevent bacterial contamination. Fluorodeoxy-uridine and cytosine arabinoside minimize glial cell proliferation, while addition of uridine triphosphate enhances neuronal survival by preventing inhibition of RNA synthesis in these non-proliferating cells (Higgins and Banker, 1998). Rat serum was added to our culture medium, as this type of serum, unlike horse or fetal bovine serum, is capable of supporting long-term survival of the cultured rat ENS (Nishi and Willard, 1985).

In initial experiments, our adult ENS cells were plated onto a collagen substrate, as is commonly performed in neonatal cultures (Nishi and Willard, 1985). However,

when subjected to experimental insult, large cell masses dislodged from the glass culture well surface on which they were grown; producing virtual sheets of cells that sloughed off of their plating surface. This cellular dissociation effectively prevented quantitation and characterization of cell injury responses. Neuronal cultures are commonly plated upon dishes coated with extracellular matrix proteins such as collagen or laminin, which markedly enhance neuronal adhesion and growth *in vitro*. During neurodegeneration, glial cells produce enzyme proteases that degrade extracellular matrix proteins such as collagen, proteoglycan, and laminin (Gottschall and Deb, 1996). This degradation raises problems for the *in vitro* study of neurodegeneration and neurotoxicity, as toxic insults are likely to induce substrate degradation and sloughing off of adherent cells; preventing evaluation and quantitation of toxicity. The use of other substrates such as the amino acid poly-D-lysine, which promotes cell adherence by altering the surface charge on the substrate (Lein et al., 1991), may circumvent this problem. However, the adhesion and maintenance of different neuronal populations varies greatly between different substrates. Thus, for use in neurotoxicological studies, a neuronal substrate that both optimally promotes cellular adhesion and maintenance, and resists degradation upon insult must be employed.

Following substrate optimization experiments, we found Matrigel matrix, a commercially available basement membrane preparation, to optimally promote cell adhesion and culture viability, and to resist digestion and degradation during cytotoxic insult. Consequently, Matrigel was selected as the substrate upon which cells were plated for neurotoxicological experiments. Fundamental requirements for the use of cell cultures in toxicological experimentation are that cell death responses be readily

identifiable and quantitable, and that specific cell death mechanisms be identifiable and distinguishable. Within these cultures, neuronal numbers were easily counted within both control and toxically insulted cultures; permitting quantitation of cell death responses. Additionally, both necrotic and apoptotic cell death mechanisms were readily identified by multiple means within our cultures; indicating the suitability of these ENS cultures for neurotoxicological study.

Our cultured enteric neurons show morphological characteristics similar to those described by Dogiel (1899) and Stach (1989) using sections and laminar preparations of the enteric nerve layers. This suggests that the shapes of neuronal cell bodies, as well as the number and morphology of their processes, are intrinsic properties of these cells that have not been altered by the culture environment. The 48% Dogiel type I, 36% Dogiel type II, 9% Stach type IV, and 7% indeterminate morphology composition present within our ENS cultures corresponds with the general morphological distribution present in the *in vivo* myenteric plexus. Although the proportions of morphological neuronal subtypes in the rat ileum have not been determined *in vivo*, within the rat colon, 50% of myenteric neurons are S type, and 40% are AH type neurons (Browning and Lees, 1996). As there is a general, though imprecise, correspondence between Dogiel type I and S type neurons, and between Dogiel type II and AH neurons (Cornelissen et al., 1999), approximately 50% of myenteric neurons in rat colon may be considered to be type I neurons, while approximately 40% may be type II neurons. Quantitation of morphological subtypes has also be performed in guinea-pig duodenum, in which 27% Dogiel type I, 54% Dogiel type II, 9% Stach type IV, and 10% unclassified neurons were identified (Lees et al., 1992). Although variations in the composition of enteric neuronal populations exist both

between species and between intestinal regions (Schemann et al., 1995; Furness et al., 1990; Ekblad et al., 1991; Wattchow et al., 1998; Sang and Young, 1996), the percentages of individual morphological subtypes observed in our ENS cultures can be considered to correspond to the general morphological subtype distribution characterized *in vivo*.

Those cells types found in our cultures are those present within *in vivo* enteric ganglia, which consist exclusively of tightly packed neurons, glial cells and their processes covered by fibroblasts and collagen fibrils (Cook and Burnstock, 1976; Gabella, 1972). The strong preference of neurons in our cultures for growth on glial cells versus fibroblasts has previously been observed in explant cultures from several species, including rat (Jessen et al., 1983). This type of selective adhesion is thought to be involved in the normal morphogenesis of the enteric plexuses.

Previous electrophysiological studies using our ENS cultures have identified that approximately 60% of neurons tested demonstrated typical AH responses and that approximately 40% demonstrated typical electrophysiological S type responses (Poulter et al., in re-submission). These *in vitro* findings resemble the 40% AH type neurons and 50% S type neurons identified *in vivo* for the rat colon (Browning and Lees, 1996). Within the present study, the electrophysiological characteristics of morphologically identified type II neurons were investigated. These neurons consistently demonstrated the single-spike, after-hyperpolarization characteristics of type II/AH neurons, and support the observed correspondence between specific morphological and electrophysiological characteristics seen for *in vivo* myenteric neurons (Browning and Lees, 1996).

The neurochemical subtypes identified in our culture correspond to those present *in vivo*, and are similar in frequency and morphological characteristics (Krantis et al., 1998, Nichols et al., 1995). In the myenteric plexus of adult rat ileum only 2.8% of neurons show VIP-like immunoreactivity, similar to the 4.1% ± 1.5% observed *in vitro*. 3.9% of myenteric neurons *in vivo* show Enk-like immunoreactivity, comparable to our finding of 4.4% ± 2.8% of total neurons expressing Met-Enk like immunoreactivity *in vitro*. In our ENS cultures, 3.7% ± 1.3% of neurons were SOM-immunoreactive, comparable to the 2% of neurons that demonstrate SOM-like immunoreactivity *in vivo* (Schultzberg et al., 1980). Although the percentage of GABAergic neurons in the myenteric plexus of rat ileum have not been quantitated, they are considered to closely correspond to the approximately 5% of total myenteric neurons that are GABAergic in guinea pig myenteric plexus (Jessen et al., 1979; Nichols et al., 1995). These numbers are similar to the 3.7% ± 1.8% of total neurons identified as GABAergic in our enteric cultures. In the myenteric plexus of adult rat ileum 16.4% of neurons display substance P like immunoreactivity (Schultzberg et al., 1980). Within our cultures, 23.5% ± 6.4% of neurons were substance P-immunoreactive. Finally, our findings that 33.5% ± 7.7% of cultured neurons are NOS-positive are also in accordance with previous findings that 24.4% (Mann et al., 1999) or 30% (Nichols et al., 1993) of all myenteric neurons in the adult rat ileum localize NOS. The correspondence of these neuronal subtype populations with those found *in vivo* indicates an absence of selective survival of particular neuronal populations in culture. Histochemical staining for nitric oxide synthase activity also demonstrates the viability and functionality of these cultured neurons.

In conclusion, we have established a method for primary culture of isolated adult rat myenteric neurons. Seven day cultures of enteric neurons reveal morphologically normal cells which demonstrate the electrophysiological and morphological characteristics, the chemical markers, and neurochemical properties of *in vivo* tissues. This cell culture protocol facilitates characterization and investigation of adult enteric neurons, and provides a physiologically relevant system for *in vitro* studies of the adult ENS. In addition, the ability to readily observe cell death and to distinguish, by multiple means, between cell death mechanisms within our neuronal cultures makes them ideal for use in neurotoxicological experiments. The quantitation of neuronal survival possible in these cultures will allow for characterization of ENS responses to toxic insult.

In this study, cryopreserved neurons were determined to be effectively identical to their freshly-plated counterparts, and we suggest their interchangeable use for experimentation. The viability of enteric neurons in culture was not significantly impaired, nor were cellular characteristics markedly altered by the cryopreservation process. For cryopreserved cells, the time course of morphological cell differentiation following plating was remarkably similar to that of freshly plated control cultures (**Chapter 2**). Additionally, those cell types present and the ratio of neurons to non-neuronal cells in culture were preserved following cryopreservation. In this study, neuronal cultures were frozen for 7 days without any quantitative changes in viability, and cells that have been frozen for 3 months thrive in culture.

Cryopreserved neurons retained those electrophysiological, morphological, and neurochemical characteristics of both freshly plated ENS cultures and their *in vivo* counterparts. The peak amplitudes, spiking limitations, and after-hyperpolarization

characteristics of the specific Dogiel type II neurons investigated within cryopreserved neurons were virtually identical to those of type II neurons both *in vivo* and in freshly plated enteric cultures (Hanani et al., 2000). The percentage composition of morphological subtypes has not been specifically determined for the rat ileum *in vivo*. However, the percentages of different morphological neuronal subtypes found in our cryopreserved ENS cultures roughly approximates those found in rat colon and guinea-pig duodenum (Browning and Lees, 1996; Lees et al., 1992), and are consistent with those found in freshly plated ENS cultures. All neurochemical subtypes investigated were determined to be present in cryopreserved cultures at concentrations highly similar to those present in both freshly plated cultures and *in vivo* (Schultzberg et al., 1980; Jessen et al., 1979; Nichols et al., 1993; 1995; Mann et al., 1999). The observation that all neuronal subtypes identified in freshly plated ENS cultures are present in similar proportions in cryopreserved ENS cultures suggests a common resistance of all enteric neuronal subtypes to the freeze-thaw stresses of this cryopreservation procedure.

Thus, we have established the viability for cryopreservation storage of isolated adult rat enteric neurons. Seven-day cultures of these frozen cells reveal morphologically normal cells that demonstrate the morphological electrophysiological and neurochemical characteristics of their freshly plated counterparts. Furthermore, there was no significant loss of neuronal viability as a result of cryopreservation. This cryopreservation procedure will be very useful for collaborative work due to the transportability of frozen cells between laboratories, and should facilitate our studies of cultured adult rat enteric neurons.

In this investigation for identification of specific inducers of ENS neurotoxicity, we provide the first direct evidence that HNE, a major product of lipid peroxidation and mediator of cellular damage associated with oxidative stress (Esterbauer et al., 1991; Chen and Yu, 1994; Chen et al., 1997; Tsukamoto et al., 1995), induces neurodegeneration in primary cultures of rat intestinal myenteric neurons. Our results indicate that both lipid peroxidation and oxidative stress mechanisms act to damage myenteric neurons, similar to their actions in CNS neurodegeneration (Bredesen, 1995; Keller et al., 1997; Blanc et al., 1997; Kruman et al., 1997; Kirchgessner et al., 1997; Mark et al., 1997).

Our experiments demonstrate the greater sensitivity of myenteric neurons to HNE insult as compared to glial cells and fibroblasts in culture. However, we also observed a subset of myenteric neurons, the NO synthesizing neurons, to be preferentially resistant to HNE-induced neurotoxicity. Interestingly, in a previous study of surgical specimens from Hirschsprung's Disease patients, workers in our laboratory observed that NO-related innervation of the human colon is uniquely unaffected in some patients, with preservation of a subpopulation of NO synthesizing myenteric neurons (Cuffari et al., 1993). Similarly, in a study of idiopathic chronic constipation tissue, the low total neuron density within the myenteric plexus contrasted with the high density of NO synthase (NOS)-positive neurons (Cortesini et al., 1995). Thus, our observation of NOS neuron resistance to toxic insult has clinical correlations in the diseased human ENS.

The properties underlying the selective resistance of enteric NOS neurons to HNE–induced damage are unclear. We observed a down-regulation of NOS activity in the myenteric NOS neurons resistant to HNE-induced apoptosis, which may represent a

temporary down-regulation of cellular functions as apoptosis-combating mechanisms are employed. Alternatively, NO production may be down-regulated as other neuronal subpopulations, which normally act as targets for NO neurotransmitter activity, degenerate in response to HNE insult. We propose that the high levels of mitochondrial manganese superoxide dismutase (MnSOD) present in enteric NOS neurons (Fang & Christensen, 1995) are most likely to provide protection against HNE-induced damage in this cell type. MnSOD acts to detoxify oxygen free radicals (Buechter, 1988), and its overexpression in cultured cortical neurons provides dramatic protection against ROS-mediated toxicity (Gonzalez-Zulueta et al., 1998).

In our experiments, we examined the involvement of the free radical NO in enteric neurodegeneration using the NO donor SNP. Due to the high instability and short half-life of NO, a variety of NO donors, which themselves have only low intrinsic toxicity, are commonly utilized to slowly release nitric oxide, and any observed effects are generally ascribed to NO itself (Beckman and Koppenol, 1996). While previous enteric nervous system studies (Hogaboam et al., 1995) have suggested NO may have some involvement in enteric neuronal damage, our findings represent the first direct evidence of a role that NO actions on the ENS induce enteric neurodegeneration in a concentration-dependent manner.

In this study, exposure of myenteric cell cultures to exogenous H_2O_2 precipitated neuronal apoptosis which was accompanied by necrosis at higher concentrations; similar to its effects in cortical and striatal cells (Bonfoco et al., 1995; Desagher et al., 1996; Whittemore et al., 1994). As seen in CNS cultures (Beckman et al., 1993; Whittemore et al., 1994), H_2O_2 had greater toxicity for neurons than for non-neuronal cells present in our

enteric cell cultures. These differences in cell-type sensitivity to H_2O_2 may be due to differing capacities of cell types to metabolize and detoxify the toxin.

In our culture system, the actions of exogenous H_2O_2 were confirmed in study of the neurodegenerative effects of endogenously-generated H_2O_2. As H_2O_2, like most ROS, has high reactivity and a short half-life, endogenous generators of H_2O_2 are commonly employed to ensure the slow generation and release of H_2O_2 into the system of interest. The neurotoxic properties of menadione, which is taken up by cells and produces superoxide anions and H_2O_2 via redox cycling (White and Clark, 1988), were similar to those of exogenous H_2O_2, except that menadione induced significantly more toxicity in neurons than could be obtained with the same concentration of H_2O_2. The greater neurotoxicity of menadione as compared to H_2O_2 may be due to interaction of the superoxide and H_2O_2, both generated by menadione breakdown to produce highly toxic hydroxyl radicals (Stark and Szurszeweski, 1992; Desagher et al., 1996).

The toxicity of NO within cells is known to be greatly enhanced by diffusion-limited reaction with superoxide (a product of H_2O_2), to produce the powerful and toxic oxidant, peroxynitrite (Beckman et al., 1990; Beckman and Koppenol, 1996). In our experiments the combined neurotoxic effects of given concentrations of H_2O_2 and the NO donor SNP were found to be significantly greater than the sum of their individual toxicities. We suggest that a diffusion-limited reaction between H_2O_2 and SNP to produce peroxynitrite in our experiments is reflected in the defined minimal concentrations of these compounds required for neurotoxicity to be potentiated, and by the increase in potentiation observed with increasing H_2O_2 and SNP concentrations.

The reactive oxygen species examined in this *in vitro* study generally induce ENS neurotoxicity at physiologically relevant concentrations. There in a paucity of data on *in vivo* ROS concentrations in the vicinity of the ENS, however, some data exists on ROS concentrations within the intestine during inflammatory or toxic injury. Although a significant body of evidence indicates that H_2O_2 plays an important role in the injury underlying several intestinal disorders (Williams et al., 1990; Halliwell et al., 1992; Harris et al., 1992), it is difficult to accurately measure H_2O_2 concentrations under physiological or pathophysiological conditions due to its very short half-life. Estimates of H_2O_2 levels produced during toxic insult to the intestine are in the order of 0.5 mM concentrations. Previous experiments have also noted that 0.5 mM – 10 mM H_2O_2 induces intestinal epithelial cell injury *in vitro* (Rao et al., 1997). Thus, our observation of H_2O_2 –induced neurodegeneration at 10 μM – 1 mM concentrations is consistent both with those concentrations at which H_2O_2 injures other intestinal cell types, and with those concentrations present during intestinal pathogenesis. Similarly, the concentrations of the NO donor SNP used in our experiments (0.1 μM – 1 mM) were in the same concentration range as those used to induce injury in isolated intestinal epithelial cells (1 μM –1 mM) (Tepperman et al., 1994; 1998).

Enteric neurons in our culture system proved to be highly resistant to damage and degeneration induced by 2% O_2 hypoxia-reoxygenation insult. Bouts of mild inflammation regularly occur within the gastrointestinal system (Collins, 1996), and tissue hypoxia is a common result of such inflammation. ENS resistance to such hypoxic insult would be advantageous for the maintenance of normal GI function in this variable environment. That ENS neurodegeneration has not been reported following such bouts

of mild intestinal inflammation is consistent with our *in vitro* observations of ENS resistance to hypoxic insult. CNS neurons also demonstrate resistance to hypoxic insult (Goldberg et al., 1987; 1988) which is attributed to multiple adaptive responses active within these cells. These neuronal adaptive responses to hypoxia include metabolic down-regulation, ionic-induced activation of O_2 receptors, and the up-regulation of certain genes; all of which enhance the systemic delivery of oxygen, promote energy conservation, and help to maintain membrane integrity (Boutilier and St-Pierre, 2000). Ion channels such as ATP sensitive K+ channels, activated by hypoxia, attenuate cellular depolarization and contribute to cellular resistance to hypoxic insult (Haddad and Jiang, 1994). Hypoxia also induces the expression hypoxia-induced expression of genes encoding such anti-apoptotic and survival factors as c-Jun, bFGF, bcl-2, and NGF, that are associated with prevention of neuronal cell apoptosis (Banasiak et al., 1999; Yun et al., 1997). Such mechanisms may also underlie enteric neuronal resistance to hypoxia, and will be investigated in further studies.

In this study, anoxic insult alone was demonstrated to induce enteric neurodegeneration, with neuronal cell loss increasing with increasing anoxic exposure duration. The reoxygenation processes following anoxia were also demonstrated to contribute to neuronal cell death, as the addition of reoxygenation periods subsequent to anoxia induced substantially greater neurodegeneration than did anoxia alone. Further studies indicated that increased anoxic exposure induced substantially less neurodegeneration than did reoxygenation of cultures for that same time period. These findings suggest that reoxygenation itself is capable of initiating cell death processes in enteric neurons, and that this reoxygenation period does not merely serve to provide

further opportunity for anoxia-induced neurodegeneration processes to occur. These findings also indicate that anoxic insult in some way alters enteric neuronal responses to normoxic conditions. Whereas neurons do not normally die in the presence of 5% CO_2, 95% air, prior O_2 deprivation renders this gaseous mixture toxic to neurons during the reoxygenation process.

Our findings of enteric neuronal resistance to hypoxia-reoxygenation insult, and marked susceptibility to injury by anoxia-reoxygenation insult are similar to those observed in cultured CNS neurons (Goldberg et al., 1987; 1988). Such similarities between anoxia-induced injury in our enteric neurons and that in CNS neurons suggest that anoxia injures enteric and central neurons by similar mechanisms, including depletion of intracellular energy sources, pH decreases to damaging acidic levels, anoxic depolarization, membrane permeability abnormalities, and increases in intracellular Ca^{2+} levels that induce neuronal damage (Maulucci-Gedde and Choi, 1987; Choi, 1987; White and Reynolds, 1997; Budd, 1998; Tombaugh and Sapolsky, 1993).

Our observations that both anoxia and subsequent reoxygenation contribute to neuronal cell death in our ENS cultures have also been noted in CNS cultures (Saikumar et al., 1998). In CNS neurons, anoxia activates multiple degradative systems to induce cell death by necrosis. Apoptosis, which is an active process requiring energy, is commonly prevented during anoxia due to depletion of cellular energy sources (Toescu, 1998; Renvoize et al., 1998). In neurons subjected to sublethal anoxic injury, restoration of aerobic glycolysis during reoxygenation replenishes cellular energy sources. Reoxygenation thus generates the energy required for apoptotic processes to occur in cells damaged by anoxic injury (Saikumar et al., 1998). However, if permanent

mitochondrial damage occurs during anoxia, impairing neuronal respiration, these cells will die necrotically due to prolonged ATP depletion either during anoxia or subsequent reoxygenation processes (Eguchi et al., 1997; Leist et al., 1997; Saikumar et al., 1998). We propose that such processes also underlie the neurodegeneration that occurs in response to anoxia and reoxygenation insults.

Glial cell viability was largely unaffected by either anoxia or anoxia/reoxygenation in our cultures, similar to that seen in CNS glial cultures (Goldberg et al., 1987; Giffard et al., 1992). Although the mechanisms underlying the resistance of glia to hypoxic damage have not been characterized in our experiments, we suggest their similarity to putative mechanisms identified within other glial populations. For example, within cultured CNS glia, up-regulation of the stress protein "protein-disulfide isomerase" is thought to play a critical role in the resistance of these cells to hypoxic and anoxic damage (Tanaka et al., 2000).

This study establishes the ability of anoxia-reoxygenation to directly induce the degeneration of enteric neurons, and suggests that such processes may be involved in the ENS degeneration associated with intestinal ischemia/anoxia in clinical disease. The marked resistance of ENS neurons to hypoxia contrasts with their high susceptibility to anoxia; suggesting that even mild relief from anoxia/ischemia may substantially reduce damage to enteric neurons *in vivo*. These findings merit further investigation of the role of intestinal anoxia/ischemia on ENS viability *in vivo* and in disease pathogenesis. Investigations of anoxic injury in the ENS are likely to benefit from the extensive experimentation performed within the CNS. With the suggested similarities between intracellular damage mechanisms in the ENS and the CNS, current therapeutic treatments

to prevent and/or attenuate CNS neurodegeneration present as likely candidate treatments for such degeneration in the ENS. Ischemic injury and enteric neurodegeneration have widespread occurrence within intestinal disease, and can produce severe damage. Elucidation of their relationship within disease pathogenesis represents the initial step towards development of therapeutic treatments for such clinical damage.

An important finding of our study with therapeutic potential was the ability of GDNF, at a concentration which reduces apoptosis in central dopaminergic neurons *in vitro* (Clarkson et al., 1995; 1997), to selectively protect cultured myenteric neurons from apoptosis induced by H_2O_2, but not that induced by anoxia-reoxygenation insult, by SNP, or by combined H_2O_2 and SNP insult. This represents the first evidence for a neuroprotective action of GDNF in adult enteric neurons. These findings suggest that GDNF may act as an endogenous neuroprotective factor in the ENS, which may have potential clinical application for the development of agents to protect against enteric neurodegeneration. However, the selectivity of GDNF neuroprotection in our study is intriguing. In the CNS, it is known that different apoptotic stimuli can invoke divergent apoptotic pathways (Taylor et al., 1997), and that for central neurons undergoing oxidative stress the survival-promoting effects of neurotrophins require activation of distinct anti-apoptotic signal transduction pathways (Skaper et al., 1998; Hetman et al., 1999). We suggest that H_2O_2 induces a separate apoptotic signal transduction pathway from those of peroxynitrite and SNP within enteric neurons. Thus, the ability of GDNF to selectively attenuate H_2O_2-induced neurodegeneration in cultured enteric neurons may be due either to GDNF interaction with a product of intracellular H_2O_2 reaction not

generated during SNP or peroxynitrite-induced damage, or to GDNF interference with that apoptotic pathway initiated by H_2O_2, but not that initiated by SNP or peroxynitrite.

In summary, our characterization of the sensitivity of cultured myenteric neurons to both early and late stage mediators of lipid peroxidation and oxidative stress establishes these processes as potential mechanisms of enteric neurodegeneration *in vivo.* The potent protection against particular oxidative stress-induced injuries in myenteric neurons by applied GDNF identifies this neurotrophin as a potential enteric neuroprotective agent. *In vivo*, GDNF is present in the adult gut wall, located primarily in the smooth muscle surrounding enteric ganglia (Peters et al., 1998), while the GDNF receptor (Ret) is found in enteric neurons but not associated glia (Bar et al., 1997). Given the *in vivo* production of GDNF in the adult intestine in close proximity to enteric neurons, and the potential for these neurons to respond to GDNF actions, GDNF represents a candidate endogenous neuroprotective agent for the adult ENS. We suggest that GDNF represents a potential therapeutic treatment for treating gastrointestinal diseases in which neuronal injury is a pathogenic feature.

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Chapter 3

Sensitization to the effects of tumor necrosis factor-α: macrophage-dependent and -independent actions of rhTNF on enteric neuron function and viability

Abstract

Degeneration of enteric neurons within the gastrointestinal tract disrupts intestinal motility, impairs intestinal absorption, and can cause weight loss and malnutrition. Clinically, ENS degeneration has been noted as a feature of inflammatory bowel disease (IBD). Current experimental therapies focus on reducing effective tumor necrosis factor-α (TNF-α) concentrations for attenuation of IBD symptoms. However, the role that TNF-α plays in inflammatory intestinal damage mechanisms is unclear, as are its actions on specific intestinal cell types including enteric neurons. This study determined that, in the absence of overt inflammation, systemic injection of low-dose rhTNF (1.5 nmoles/kg) induced functional alterations in the murine intestine including epithelial apoptosis, stem cell activation, crypt cell proliferation, and neurochemical alterations in enteric neurons. *In vitro* studies confirmed these actions of rhTNF on enteric neurons, and demonstrated that, in the presence of macrophages, comparable (1.5nM) rhTNF doses induced substantial degeneration of enteric neurons. These results indicate that the ability of rhTNF to injure the intestinal mucosa and alter enteric neuronal function is independent of inflammatory macrophage infiltration, whereas the ability of this cytokine to induce enteric neurodegeneration is macrophage dependent. rhTNF also demonstrated the ability to sensitize intestinal tissues to the effects of further exposure to this cytokine. This self-sensitization phenomenon displayed both a time-dependency of action and regional specificity within the murine intestine. These findings further knowledge of TNF-α actions in the gastrointestinal tract, and are of direct relevance to the therapeutic targeting of TNF-α in inflammatory GI diseases.

Introduction

Tumor necrosis factor-α (TNF-α) is an important mediator of inflammatory bowel disease (IBD) pathogenesis, as increased concentrations of this cytokine maintain and amplify the inflammation characteristic of IBD (Murch et al., 1993). The correlation between elevated TNF-α production and IBD pathogenesis has been described in several animal models employing specific neutralizing antibodies and cytokine gene knockouts (Neurath et al., 1997; Powrie et al., 1994; Watkins, 1997), as well as in human IBD patients (Hadziselimovic et al., 1995; Gardiner et al., 1995). Most convincingly, a critical role for TNF-α in the pathophysiology of human IBD has been demonstrated in clinical trials that report the effectiveness of a single dose of anti-TNF monoclonal antibody in healing patients with treatment-refractory IBD (Targan et al., 1997; Neurath et al., 1997; Derkx et al., 1993; Van Dullemen et al., 1995; Murthy et al., 1999). However, the exact role of TNF-α in inflammatory intestinal damage has not been fully elucidated, and there is a paucity of information on specific TNF-α actions at the cellular level within the intestine. It is particularly unclear whether TNF-α merely induces intestinal inflammation that then effectuates intestinal damage, whether TNF-α itself damages intestinal tissues, or whether TNF-α acts in conjunction with inflammatory cells to damage intestinal tissues during disease pathogenesis. In this study, we intend to investigate the specific actions of elevated TNF-α concentrations, in the absence of overt inflammation, on intestinal tissues, and to determine the involvement of inflammatory cells such as macrophages in these TNF-α actions.

Functional alterations, axonal necrosis and ganglion cell degeneration in the enteric nervous system (ENS) can occur within IBD (Dvorak, 1990; Steinhoff et al.,

1988). To date, mechanisms of enteric neurodegeneration within IBD have not been identified. However, TNF-α, which has been shown to alter neurotransmitter expression in the ENS (Hurst and Collins, 1994), and thereby affect enteric neuronal function, has been identified as a putative mediator of altered ENS function during intestinal inflammation, and may also be involved in inflammatory degeneration of the ENS. Indeed, many of the motility disturbances of IBD are hypothesized to result from TNF-α disruption of enteric neurotransmission (Hurst and Collins, 1993) and/or cytokine induced enteric neural damage (Collins, 1996). A focus of this paper will be the elucidation of TNF-α actions, both macrophage-dependent and –independent, on enteric neuronal function and viability.

Several chemical agents including glucocorticoid receptor antagonists, cytokines, galactosamine, lipopolysaccharide, and some bacterial infections are known to pre-sensitize cells and tissues to recombinant human TNF-α (rhTNF) toxicity (Brouckaert et al., 1992; Lehmann et al., 1987; Everaerdt et al., 1989; Rothstein and Schreiber, 1988; Matsuura and Galanos, 1990). We have recently shown that rhTNF also sensitizes hormone levels, central neurotransmitter activity, and animal behavior to the effects of further rhTNF exposure (Hayley et al., 1999). Gastrointestinal studies suggest that sensitization processes also occur within these tissues. These findings indicate that initial injury may sensitize intestinal tissues to the effects of further gastrointestinal insults, and that cytokine actions may be involved in this increased gut susceptibility to injury. For example, in rat models of ulcerative colitis, previous colitis renders the colon more susceptible to the effects of stress on enteric nerve function and increases some parameters of inflammation (Collins et al., 1996). Additionally, the nature of gut

inflammation (acute vs. chronic) and the frequency of cytokine exposure influences intestinal reactions to inflammatory cytokines such as IL-1 and TNF-α, with initial reactions differing from subsequent reactions (Kojouharoff et al., 1997). We hypothesize that, as in CNS tissues, TNF-α sensitizes gastrointestinal tissues to the effects of further TNF-α exposure. We intend to investigate this proposed phenomenon in the context of our studies of TNF-α actions on intestinal tissue, and on enteric neurons in particular. Understanding the specific effects of pro-inflammatory cytokine exposure and re-exposure upon the intestine would provide important information for the development of therapeutic strategies to treat inflammatory gastrointestinal diseases.

Materials and Methods

Animals

Male CD-1 mice, 8-10 weeks of age and approximately 40 g in weight, were obtained from Charles River (Laprairie, Quebec, Canada). Animals were maintained on a 12 hour light/dark cycle, and mice were permitted an *ad libitum* diet of Ralston Purina (St. Louis, MO) mouse chow. All experiments performed were approved by the Carleton University Committee for Animal Care and met the guidelines set out by the Canadian Council on Animal Care.

TNF injection protocol

Mice were transferred from the colony holding area to the testing area 24 hrs prior to initial or second injections to permit acclimatization to the testing environment. Animals received an initial intraperitoneal (ip) injection of 400 µl volume, composed of either sterile, non-pyrogenic physiological saline, or saline plus 4 µg of rhTNF (specific activity, 1.1×10^5 U/µg) (R & D Systems). A varied determinate amount of time (1 day, 7 days, 14 days, or 28 days) elapsed before mice were given a second ip injection of 400 µl volume, consisting of either saline or saline plus 1 µg of rhTNF (1.5 nmoles/kg total rhTNF added in rhTNF/rhTNF treatments). These experimental rhTNF/rhTNF treatments (T/t) and experimental controls including initial and second injections of saline (S/S), initial injections of saline with second injections of saline plus rhTNF (S/t), and initial injections of saline plus rhTNF with second injections of saline (T/S), were performed for the different injection time intervals. Mice were sacrificed by decapitation 1, 3, 8, or 24 hrs following second injections. One cm segments of duodenum, jejenum, ileum, and colon were extracted from animals and placed in modified Zamboni's fixative

consisting of 4% paraformaldehyde and 0.4% picric acid in 0.16 M phosphate buffer (PB), pH 6.9 for a 90 min. fixation. Tissues were then rinsed and stored at 4°C in 10% sucrose, 0.01% Na azide in 0.1 M PB (pH 7.2) until sectioned.

Immunohistochemistry

Horizontal and cross-cut sections (14 μm thick) of intestine were cryostat-sectioned and thaw mounted onto coated slides. A standard protocol was followed during all immunohistochemical staining in which primary antibody was diluted to its working concentration in 0.3% Triton-X100 in PBS, and applied to slides overnight at 4°C. Slides were then rinsed in 10 mM PBS, and incubated for 35 min. at 37°C with secondary antibody diluted to the appropriate concentration in 0.3% Triton-X100 in PBS. Slides were again rinsed in 10 mM PBS and mounted under a fluorescence fade-retarding mounting medium (0.1% *p*-phenylenediamine, pH 7.0, 10mM PBS, 692 g/l glycerol at pH 7.8). Immunohistochemical staining was performed in tissues from a minimum of five animals for each experimental treatment.

All immunohistochemicals used in these procedures were commercially available antibodies of known specificity unless otherwise noted. Prior to use, staining characteristics and specificity of each antibody were tested and confirmed by staining in control tissues with established staining patterns. Polyclonal anti-heme oxygenase 1 (HO-1; 1:1000; StressGen), and FITC-conjugated donkey anti-rabbit IgG (1:20; Amersham), were used to visualize HO-1 activity. Anti-IL-1α and anti-IL-1β antisera were a kind gift of Dr. M. Schultzberg (Karolinska Institute, Stockholm, Sweden). Polyclonal anti-IL-1α (1:400 dilution) and anti-IL-1β (1:500 dilution) were both used in conjunction with a secondary Cy3-conjugated donkey anti-rabbit IgG (1:100; Chemicon).

Polyclonal anti-macrophage (1:200; Accurate Chemicals), and FITC-conjugated donkey anti-rabbit IgG (1:20; Amersham), were used to visualize this cell type. Double staining using two rabbit polyclonal antibodies was performed using polyclonal anti-thymidylate synthase antibody kindly donated by Dr. Birnboim (University of Ottawa, Ottawa, Canada) (1:400 dilution) and polyclonal anti-c-fos antibody (Cambridge Research Biochemicals Ltd) (1:1000 dilution) employing a commercial Fab antibody kit (Jackson ImmunoResearch).

TUNEL

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) staining was performed for identification of early apoptotic cells as per manufacturer's protocols (Boehringer Mannheim).

Toluidine blue histochemistry

Toluidine blue staining is used to identify mast cells within tissues by the characteristic blue/purple staining of this cell type. In this staining procedure, 14 μm thick tissue sections were rinsed three times in 0.66 M HCl, then immersed overnight in acid toluidine blue solution [6.25 g/L toluidine blue (Sigma) in 0.66 M HCl]. Tissue sections were then thoroughly rinsed in 0.66 M HCl prior to dehydration and mounting in permount.

ENS cultures - Tissue Isolation

Male Sprague Dawley rats 200-250 g (Charles River laboratories, Canada) were sacrificed by cervical dislocation according to Canadian Council on Animal Care guidelines, and a 30 cm segment of distal ileum was isolated. This tissue was flushed with freshly-prepared oxygenated Kreb's solution (7.36 g/L NaCl, 0.385 g/L KCl, 0.166

g/L $NaH_2PO_4(H_2O)$, 0.245 g/L $MgCl_2.6H_20$, 0.368 g/L $CaCl_2.2H_2O$, 2.0 g/L glucose, and 2.1 g/L $NaHCO_3$) to remove intestinal contents, and tissue was maintained in continuously-oxygenated Kreb's solution on ice until processed for dissection. Two cm segments of ileum were cut longitudinally and pinned flat, mucosal side up. Throughout dissection, tissue was maintained in continuously-oxygenated Kreb's solution. Under a dissecting microscope, villi were scraped away from underlying muscle layers. Mucosa and circular muscle, as a single layer, were then separated from the myenteric plexus and longitudinal muscle layers underneath. Remaining circular muscle fibres were removed. The resulting 'laminar preparations' were maintained on ice until processed for enzymatic dissociation of cells.

Enzymatic Dissociation of Cells

Isolated laminar preparations of myenteric plexus/longitudinal muscle were enzymatically dissociated. Isolated tissue was placed in 10 ml of filter-sterilized Hanks Balanced Salt Solution pH 7.3 (Gibco BRL) containing 100 µl of papain II (Worthington) and 1.0 mg of L-cysteine (Sigma), and then incubated for 10 min. in a 37°C water bath. This tissue was rinsed in a L-15 washing medium composed of Leibovitz's L-15 medium (Gibco BRL) with 10% fetal bovine serum (Gibco BRL), 2mM L-glutamine (Gibco BRL), 15 mM glucose (BDH) and 100U penicillin-streptomycin (Gibco BRL). After rinsing, tissue was incubated in a 37°C water bath for 10 min in 10 ml of Hanks Balanced Salt Solution (Gibco BRL) containing 3000U of collagenase type I (Worthington) and 20U of dispase II (Boehringer Mannheim). Tissue in this solution was then triturated with a flame-polished Pasteur pipette until cells were dissociated (approximately 40 triturations). This medium was centrifuged at 4°C for 5 min. at 500 rpm, and the pellet

resuspended in 8ml of L-15 washing medium. Centrifugation was repeated for 5 min. at 500 rpm and 4°C, and the pellet was resuspended in feeding medium composed of 90% MEM (Gibco BRL), 2.5% rat serum (Gibco BRL), 2 mM L-glutamine, 15 mM glucose, 100U penicillin-streptomycin (Gibco BRL), 10 μM fluorodeoxy-uridine, 1 mM cytosine arabinoside and 0.5 mM uridine triphosphate.

Cell Plating

Cell suspensions were plated at an approximate concentration of 570 cells / mm^2 onto sterile glass LabTek 8 well chamber slides (VWR Canlab) coated with Matrigel matrix (VWR Canlab). Cultures were maintained at 37°C in an incubation chamber (gassed with 5% CO_2 in air), and feeding medium was changed after the initial 24 hr incubation and every 72 hrs subsequently. Cultures were generally used in experiments seven days after plating.

ENS-macrophage co-culture experiments

The murine macrophage cell line RAW 264.7 (ATCC, AR) was harvested and plated according to the supplier's stipulations, and was maintained in a Dulbecco's Modified Eagle Medium-based solution following the protocol of Brana et al. (1999). Five days following plating of ENS cultures, defined numbers of macrophages were added to ENS cultures. These RAW 264.7 macrophages, which were harvested from their respective cultures, were pelleted, resuspended in a minimal volume of ENS culture medium, and added to ENS cultures at an approximate ratio of 2 macrophages: 5 neurons. Macrophages were permitted 24 hrs to adhere to culture substrate prior to culture medium replacement with fresh medium. Macrophages and neurons were co-cultured for 48 hrs prior to commencement of experimental testing.

In vitro rhTNF neurotoxicity

The *in vitro* toxicity of rhTNF for enteric neurons was tested on 7 day ENS cultures using comparable effective doses of rhTNF to those used *in vivo*. In this experiment, 0.1 μg/ml of rhTNF was added to 7 day cultures, followed after 24 hrs by a second exposure to 0.025 μg/ml of rhTNF (1.5 nM total rhTNF added in rhTNF/rhTNF treatments). Cultures were fixed 24 hrs following this second rhTNF exposure and neuronal survival quantitated. Additional experiments using up to 1000 X rhTNF concentrations confirmed the inability of rhTNF to directly induce enteric neurodegeneration.

In these experiments, 0.1 μg/ml of rhTNF was added to 7 day macrophage-ENS co-cultures, followed after 24 hrs by a second exposure to 0.025 μg/ml of rhTNF. Cultures were fixed 24 hrs following this second rhTNF exposure and neuronal survival quantitated. Control experiments determined the toxic effect of macrophages themselves on enteric neuronal survival. In these controls, neuronal viability was assessed following macrophage-ENS co-culture in the absence of any rhTNF exposure. The role of rhTNF sensitization in macrophage mediation of rhTNF-induced enteric neurodegeneration was also assessed in controls in which co-cultures were exposed to either 0.1 μg/ml rhTNF 48 hrs prior to fixation (T/S) or 0.025 μg/ml rhTNF 24 hrs prior to fixation (S/t).

In all experiments in which rhTNF neurotoxicity was quantitated *in vitro*, treated cultures were fixed for 60 minutes in picric acid/paraformaldehyde fixative (as detailed previously). Immunohistochemical staining was then performed to permit identification of neurons using polyclonal anti-neuron-specific enolase (NSE; 1:800; Dako Corp.) and Cy3-conjugated donkey anti-rabbit IgG (1:100; Jackson ImmunoResearch Inc.).

Neuronal Quantitation

Cultured neurons were visualized under fluorescent conditions with anti-neuron-specific enolase antibody, and neuronal numbers in each culture well were counted manually. Neurons actively undergoing apoptosis, as indicated by Hoechst 33258 staining, were deducted from numbers of surviving cells. Toxicity was expressed in terms of total numbers of viable neurons per experimental well.

Statistics

Unless otherwise noted, data are expressed as the mean of n=5 independent experiments. Significant differences in survival between multiple cell groups were determined using one-way ANOVA followed by post-hoc Tukey/Kramer analysis. Significance was considered established at p values of less than 0.01.

NOS histochemistry

NADPH-diaphorase histochemical staining for nitric oxide synthase (NOS) activity within cultures was performed according to published methods (Nichols et al., 1994). Fixed cultures were rinsed in 10 mM PBS, NO synthase reaction medium [1 mM NADPH, 0.5 mM nitroblue tetrazolium, 0.3% Triton X-100 in 10 mM PB (pH 8.0)] was applied, and culture slides incubated in a dark, moist chamber at 37°C for 1 hr. Slides were then rinsed in 10 mM PBS for 15 min., and in decreasing concentrations of 100 mM PB, 50 mM PB, 25 mM PB, and 12.5 mM PB for 1 min. each. They were then air-dried and coverslipped under Tris-Glycerol 1:3. The histochemical product for NADPH-dependent diaphorase, indicating NOS activity, was photographed under bright-field conditions.

Quantitation of histochemistry and immunohistochemistry

Grayscale value quantification of the intensity of NADPH diaphorase staining and IL-1 immunofluorescence, was performed using UTHSCSA ImageTool (version 2.00). For NADPH histochemistry, increasing grayscale values are indicative of decreasing staining intensity, while for IL-1 immunofluorescence, increasing grayscale values are indicative of greater immunopositivity. For evaluation of NOS activity, data are expressed as the mean of 5 experimental culture wells for each experimental treatment. Within each well, NADPH intensity was measured in a minimum of 15 macrophages. In the evaluation of IL-1α immunofluorescence intensity, stained tissues from 5 separate animals for each experimental treatment were examined. In tissue from each animal, IL-1α intensity was measured at 10 points within stained enteric neurons and at 10 points within adjacent "control" unstained smooth muscle. Significant differences between experimental groups was determined using one-way ANOVA followed by post-hoc Tukey/Kramer analysis. Significance was considered established at p values of less than 0.01.

Results

Lack of distinguishable inflammatory cell infiltration within intestinal layers

Toluidine blue staining revealed a small resident population of mast cells within the intestinal mucosa and villi in control animals. Similarly, antibody staining for macrophages revealed this cell type to be present primarily within the intestinal mucosa, submucosa, and, to a far lesser extent, between the circular and longitudinal muscle layers in control tissues. Experimental T/t treatment did not induce any discernable infiltration of either mast cells or macrophages into the intestinal wall at any experimental time point examined (between 1 and 24 hrs following a second ip injection of rhTNF) (Fig. 31).

Mucosal Responses to rhTNF treatment

rhTNF treatment produced changes in cytological markers within mucosal cells as early as 1 hr following a second ip injection of this cytokine. Within control tissues, TUNEL labeling of apoptotic cells was observed in a few scattered cells within villi (Fig. 32A). 1 hr following a second ip injection of rhTNF in T/t-treated animals, there was a marked increase in the number of apoptotic (TUNEL-labeled) goblet and epithelial cells within the mid- and upper portions of villi (Fig. 32D). Immunohistochemical staining for the presence of thymidylate synthase, an indicator of active cell division (Chu and Allegra, 1996), showed low levels of staining in the intestinal crypts of control tissues (Fig. 32B). Following double injections of rhTNF (T/t), the numbers of crypt cells labeled with thymidylate synthase increased dramatically (Fig. 32E). Finally, within control tissues, c-fos, a marker of cell activity, was observed immunohistochemically

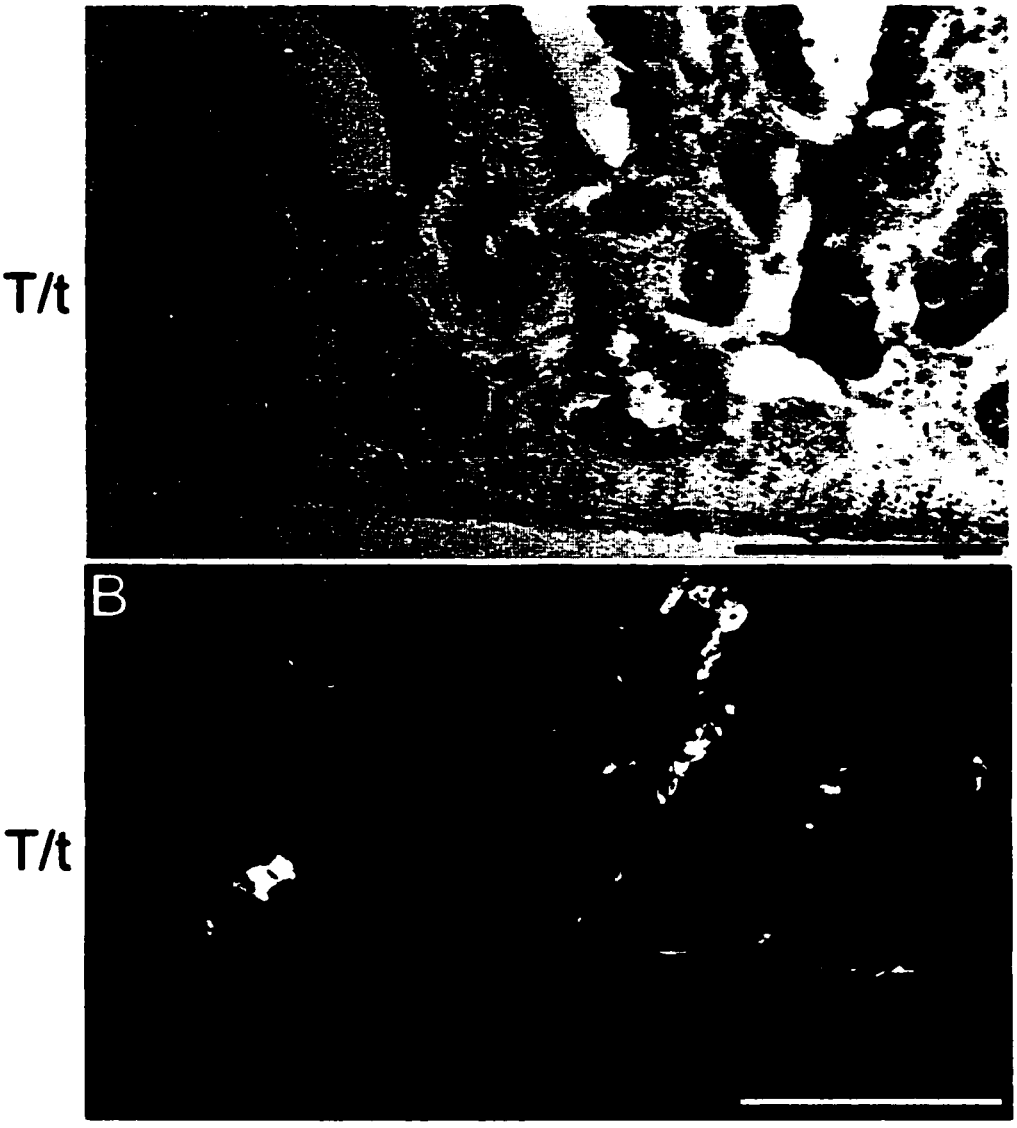

T/t
B
T/t

Figure 31. No discernable inflammatory cell infiltration occurs within murine intestine following experimental rhTNF treatment. Toluidine blue staining of mast cells (**A**) shows only occasional cells present within villi epithelium of rhTNF-treated mice (T/t treatment), indistinguishable from resident mast cell populations in S/S control treatments. Similarly, anti-macrophage staining following T/t treatment (**B**) indicates macrophage localization primarily to mucosal and submucosal layers, and to a much smaller extent, between circular and longitudinal muscle layers, as seen in controls. For all experimental rhTNF treatments (T/t, S/t, and T/S), these cell populations were indistinguishable from those in S/S control tissues. Bars represent 100 microns.

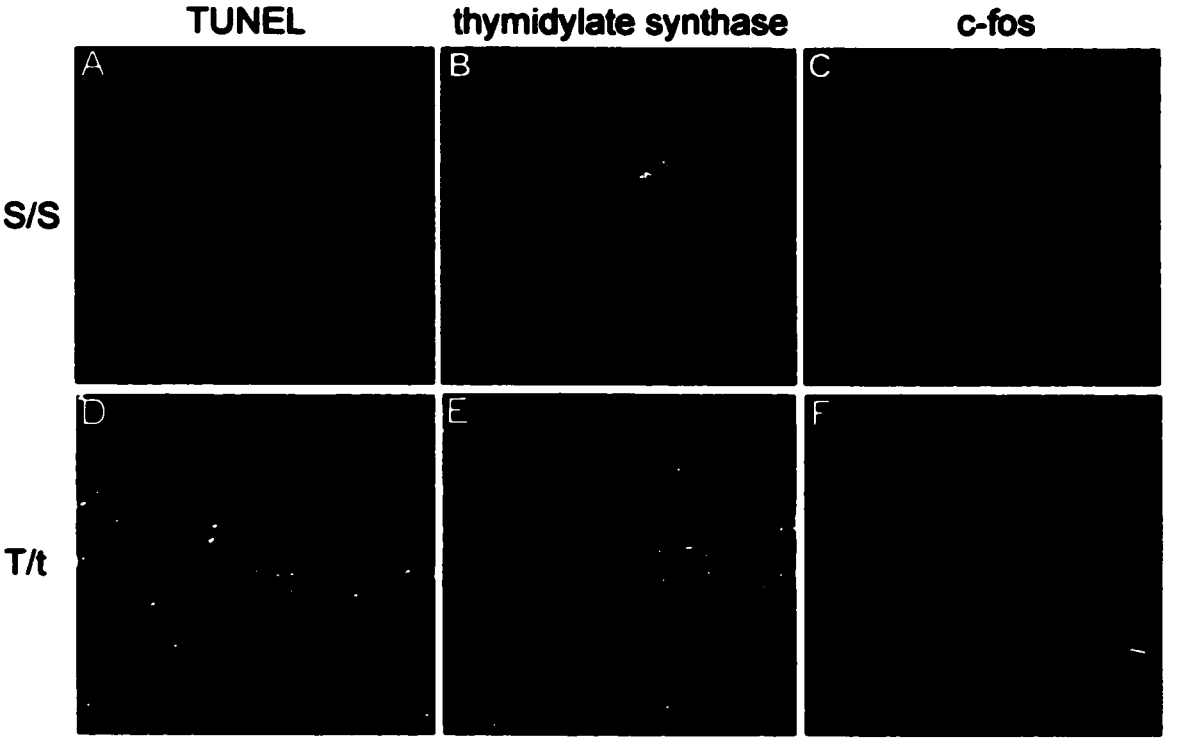

TUNEL
thymidylate synthase
c-fos
A
B
C
S/S
D
E
F
T/t

Figure 32. rhTNF-treatment induces chemical changes within the mucosa of murine ileum. Within S/S control ileal tissues, low numbers of apoptotic cells are present, as evidenced by TUNEL staining (**A**). In contrast, high numbers of actively apoptotic villi epithelial cells are present 1 hr following injection of 1μg rhTNF in TNF-sensitized animals (T/t treatment) (**D**). Low levels of thymidylate synthase activity are present in S/S control mucosa (**B**), whereas T/t exposure induces marked thymidylate synthase immunoreactivity within mucosal crypts (**E**). C-fos staining is absent in control mucosal tissues (**C**), but is induced within the base of mucosal crypts in response to T/t treatment (**F**). Double labeling of tissues for thymidylate synthase and c-fos activity (as shown in **B** & **C** and **E** & **F** respectively) demonstrates that there is no overlap in the cellular localization of these proteins. Based upon the known distribution of specific cell types within intestinal crypts, thymidylate synthase is thought to be confined to newly-formed epithelial cells within crypt walls, while c-fos is thought to be localized to stem cells present at the base of crypts. Bars represent 50 microns.

within only occasional mast cells of the intestinal mucosa (Fig.32C). T/t treatment induced marked anti-c-fos staining within cells at the base of intestinal crypts 1 hr following a second ip rhTNF injection (Fig. 32F). Double staining demonstrated that completely separate populations of cells were stained for c-fos and thymidylate synthase; anti-c-fos staining was localized to stem cells at the base of crypts, while thymidylate synthase was confined to newly-formed epithelial cells (Figs. 32E & 32F).

Neuronal Responses to rhTNF

Enteric neurons showed cytological changes as early as 1 hr following a second injection of rhTNF in experimental T/t treatments. Heme oxygenase-1 (HO-1), a rapidly induced indicator of oxidative stress (Maines, 1988), was essentially undetectable within control tissues, with only a few immunopositive mast cells observed within villi and mucosal layers (Fig. 33). T/t treatment induced anti-HO-1 immunoreactivity within neuronal cell bodies and processes of the myenteric plexus and occasional neurons of the submucosal plexus (Fig. 33). Within control tissues, positive staining for the presence of the pro-inflammatory cytokine interleukin-1α (IL-1α) was restricted to small numbers of villi epithelial cells. T/t treatment induced expression of IL-1α within large numbers of neuronal cell bodies and processes of the myenteric plexus, and within a small number of neurons of the submucosal plexus 1hr following a second ip rhTNF (T/t) treatment. Immunohistochemically detectable changes in IL-1β expression were not observed, however.

Within S/S control cultures of myenteric neurons from adult rat ileum, positive staining for the presence of IL-1α was confined to a small subpopulation of enteric neurons (Fig. 33). Similar staining profiles were seen in control cultures treated with

single doses of rhTNF (S/t and T/S treatments) (Fig. 33). In T/t treated cultures, intense IL-1α expression was induced in a majority of enteric neurons. For all culture treatments (S/S, S/t, T/S, and T/t), cultured ileal enteric neurons did not demonstrate any positive staining for the presence of HO-1.

Sensitization of the intestinal mucosa and enteric neurons to rhTNF

Both intestinal mucosal cells and enteric neurons showed sensitization to a second injection of rhTNF (T/t treatment). By these means, otherwise sub-effective doses of rhTNF produced dramatic changes within cells (Fig. 34). Tissues exposed to single doses of rhTNF in both T/S and S/t controls did not differ from saline controls (S/S). However, when the initial sub-effective dose of rhTNF was followed by a second much smaller dose (T/t treatment), dramatic cellular responses occurred. This sensitization of mucosal cells and enteric neurons to the effects of rhTNF was dependent upon the time interval between initial and subsequent rhTNF injections (Fig. 35). Sensitization decreased with the length of time between injections; all cellular responses were maximal at 1 and 7 day intervals between rhTNF injections, reduced at 14 day intervals, and undetectable at 28 day intervals between injections (Fig. 35).

Regional variations in cellular responses to rhTNF

The observed cellular responses to rhTNF injection were confined to specific intestinal regions (Table 3). In contrast to the large number of cellular changes within the distal ileum, there was a limited effect of rhTNF treatment (T/t) in the jejunum, and no cellular changes were detected within duodenum. Those regions responding to rhTNF treatment varied dependant upon which specific cellular response was being examined. In tissue sections, rhTNF induced IL-1α expression within enteric neurons of both ileum

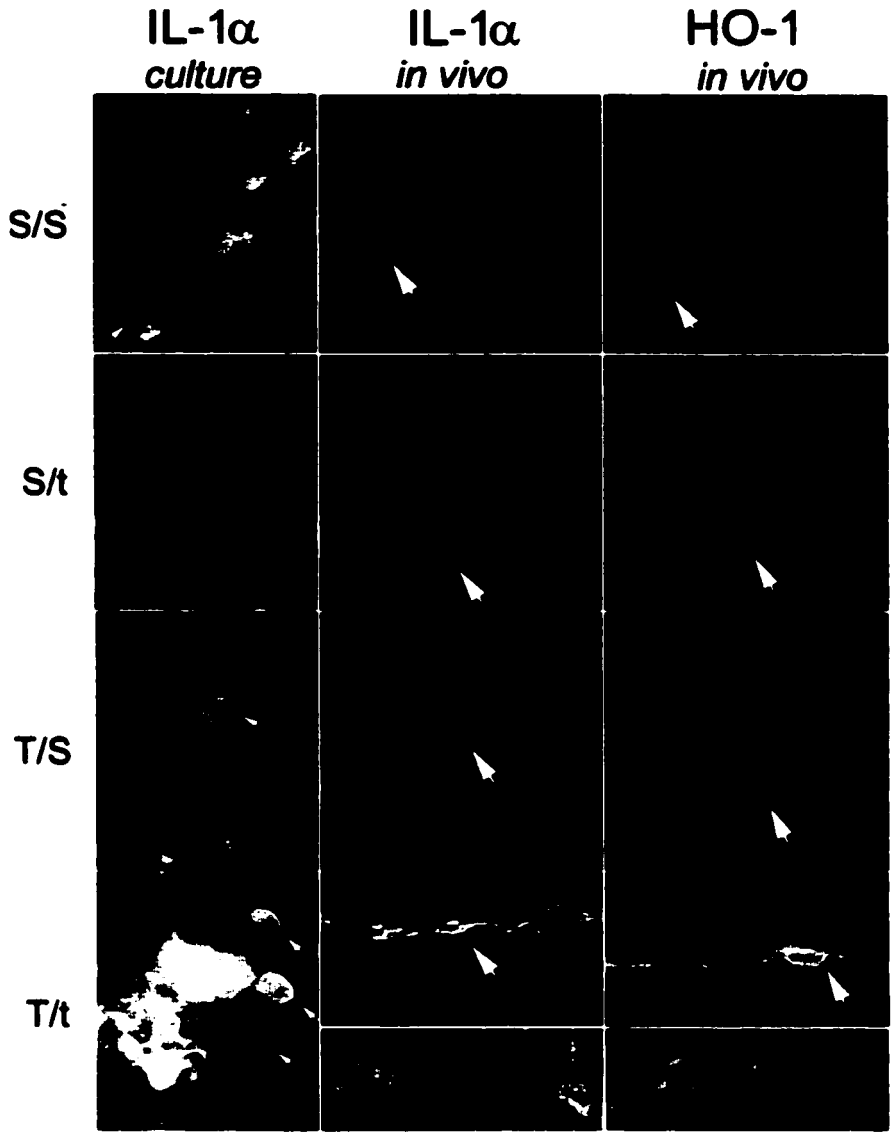

IL-1α
culture
IL-1α
in vivo
HO-1
in vivo
S/S
S/t
T/S
T/t

Figure 33. Sensitization of enteric neurons to rhTNF by low dose preexposure to rhTNF. Injection of either saline alone **(S/S treatment),** 0.3 nmoles/kg rhTNF **(S/t treatment)** or of 1.2 nmoles/kg rhTNF **(T/S treatment)** does not induce detectable changes in either anti-IL-1α or anti-HO-1 immunoreactivity within enteric neurons of the murine intestine. In constrast, injection of 1.2 nmoles/kg rhTNF followed after 1-14 days interval by an injection of 0.3 nmoles/kg rhTNF **(T/t treatment)** induces marked immunoreactivity for both IL-1α and HO-1 in enteric neurons of the murine colon. Arrows indicate enteric ganglia within the intestinal wall. Within T/t treated tissues stained for IL-1α and HO-1 immunoreactivity, both horizontal and cross-cut intestinal sections are used to illustrate the staining characteristics of immunoreactive myenteric neurons. In culture, saline, 0.3 nM or 1.2 nM rhTNF treatments **(S/S, S/t, T/S)** have no effect on IL-1α immunoreactivity within enteric neurons. However, treatment with 1.2 nM rhTNF followed 1 day later by treatment with 0.3 nM rhTNF increases IL-1 α immunoreactivity within ENS cultures from adult rat ileum. Bars represent 50 microns.

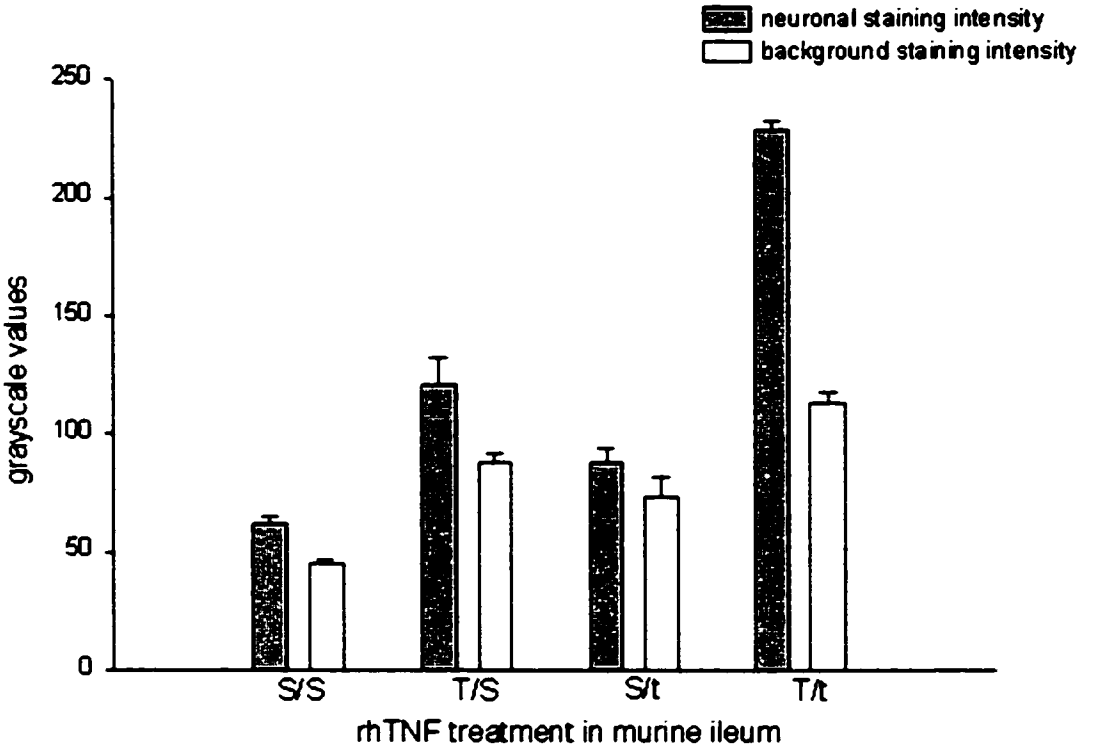
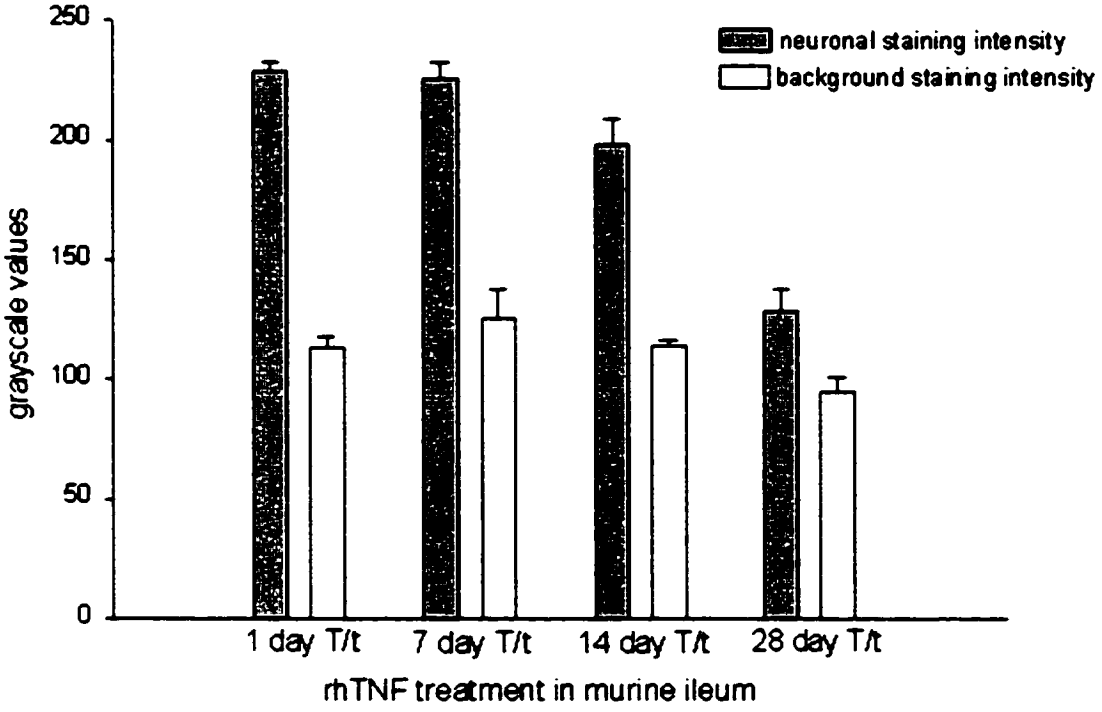

neuronal staining intensity
background staining intensity
250
200
150
100
50
0
grayscale values
S/S
T/S
S/t
T/t
rhTNF treatment in murine ileum

Figure 34. Sensitization of enteric neurons by initial rhTNF exposure to the effects of subsequent rhTNF exposure. Injection of 1.2 nmol/kg rhTNF (T), followed after 1 day by a second injection of 0.3 nmol/kg rhTNF (t), markedly increased immunoreactivity for the presence of IL-1α in enteric neurons of murine ileum. IL-1α immunoreactivity in T/t treated sections was compared to that of S/S, S/t, and T/t controls. Increasing arbitrary grayscale levels indicate an increase in intensity of IL-1α immunofluorescence.


250
200
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50
0
grayscale values
neuronal staining intensity
background staining intensity
1 day T/t
7 day T/t
14 day T/t
28 day T/t
rhTNF treatment in murine ileum

Figure 35. Time dependence of the self-sensitization to rhTNF. Injection of 1.2 nmol/kg rhTNF (T) was followed at various time intervals by a second injection of 0.3 nmol/kg rhTNF (t). The marked increase in immunoreactivity for the presence of IL-1α in enteric neurons of murine ileum in response to this T/t treatment decreased with increasing time intervals between these two rhTNF injections. Increasing arbitrary grayscale levels indicate an increase in intensity of IL-1α immunofluorescence.

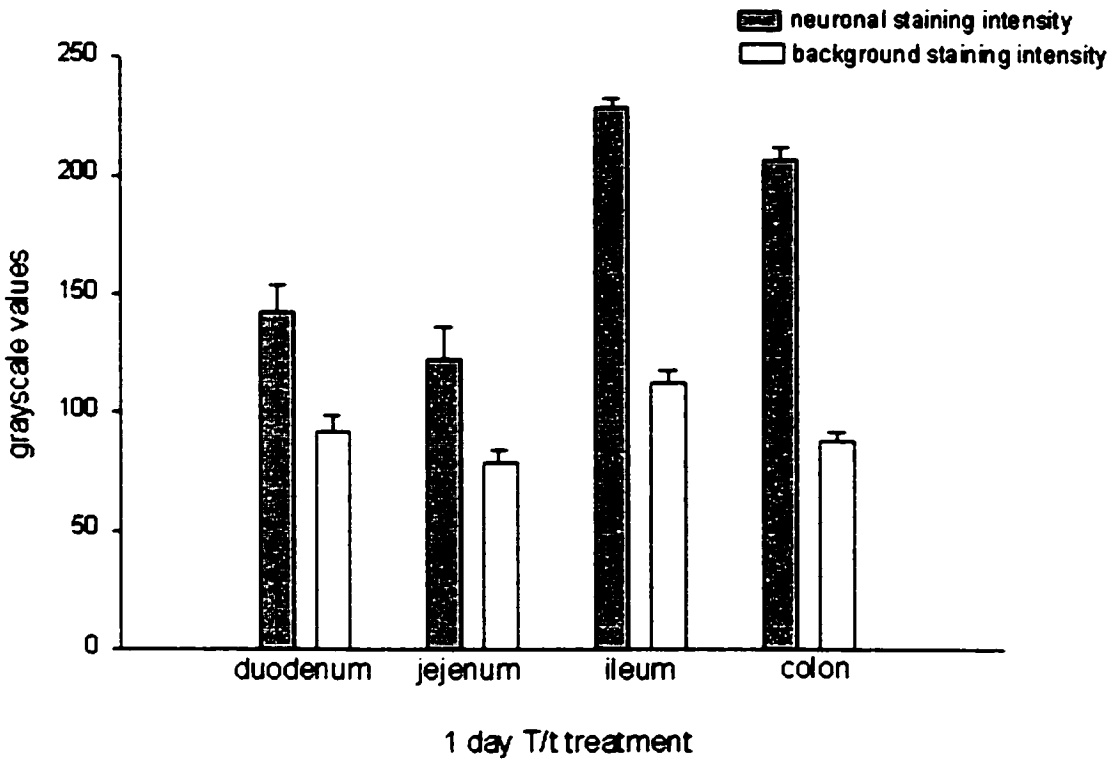
neuronal staining intensity
background staining intensity
250
200
150
100
50
0
grayscale values
duodenum
jejenum
ileum
colon
1 day T/t treatment

Figure 36. Enteric neurons demonstrate regional variations in their sensitization to rhTNF treatment. Injection of 1.2 nmol/kg rhTNF (T) followed after 1 day by a second injection of 0.3 nmol/kg rhTNF (t), markedly increases immunoreactivity for the presence of IL-1α in enteric neurons in murine ileum and colon, but not in the duodenum or jejunum. Increasing arbitrary grayscale levels indicate an increase in intensity of IL-1α immunofluorescence.

rhTNF-α effects on neuronal survival in ENS cultures and macrophage-ENS co-cultures

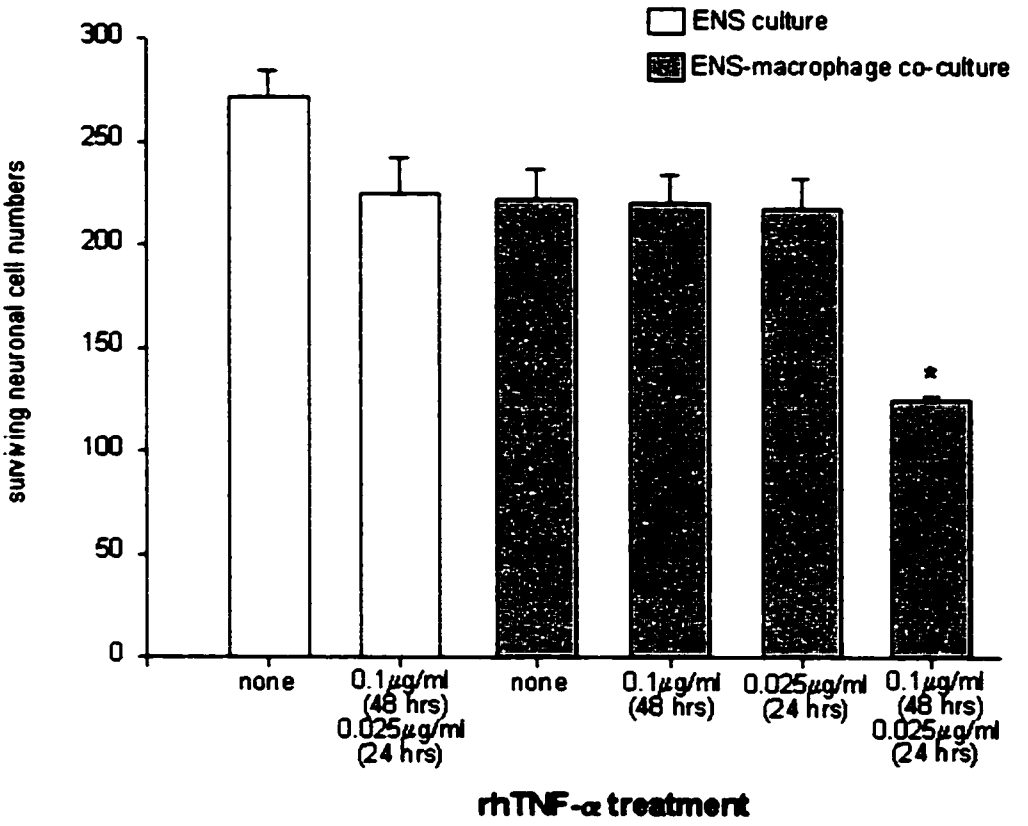

Figure 37. Determination of the role of macrophages in rhTNF-induced neurotoxicity. No significant neuronal cell death is induced within ENS cultures by rhTNF treatment (S/t, T/S, or T/t treatments). No significant neuronal cell death is induced within ENS cultures by co-culture of enteric neurons with macrophages. While neuron/macrophage co-cultures are insensitive to single dose, non-sensitized exposure to rhTNF (either S/t or T/S treatments), significant and substantial neuronal cell death results from rhTNF treatment in rhTNF sensitized co-cultures (T/t treatment). ENS cultures were isolated from the myenteric plexus of adult rat ileum, and cultured for 7 days prior to initial 0.1μg/ml rhTNF exposure 48 hrs before fixation. For T/t treatment, cultures were subsequently exposed to 0.025μg/ml rhTNF 24hrs prior to fixation. In ENS-macrophage co-cultures, macrophages of the murine RAW 264.7 macrophage cell line were added to 5 day ENS cultures in a 2:5 macrophage: neuron ratio, and co-cultured for 2 days prior to any rhTNF exposure. All experimental cultures were fixed following 9 days of culture. * $p<0.01$ (one-way ANOVA followed by post-hoc Tukey/Kramer analysis).

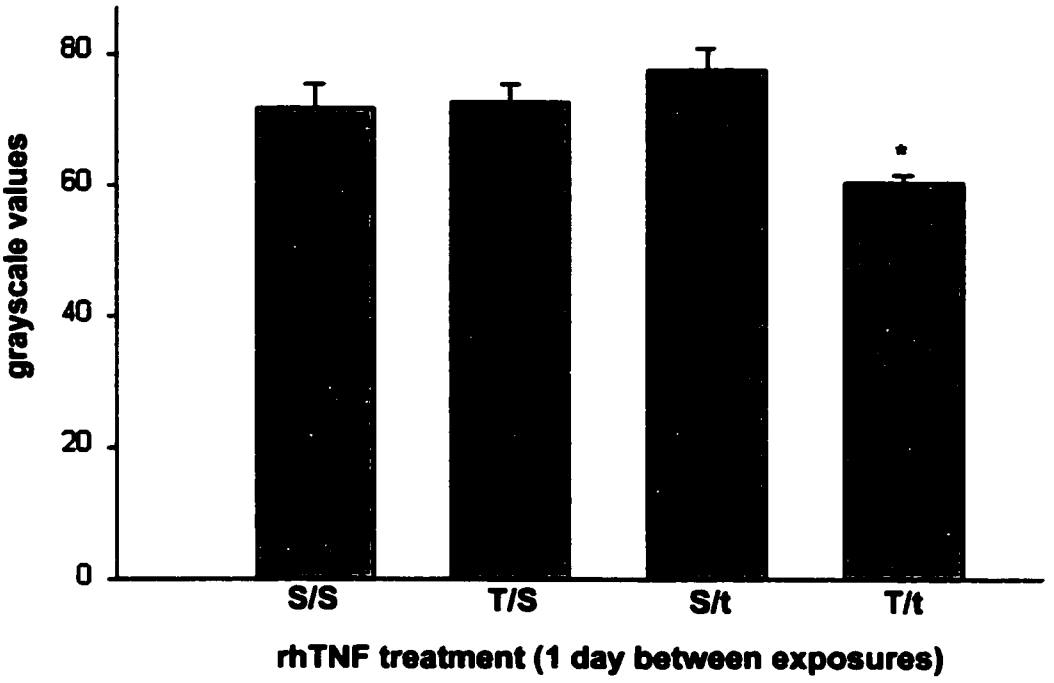
80
60
40
20
0
grayscale values
*
S/S
T/S
S/t
T/t
rhTNF treatment (1 day between exposures)

Figure 38. Sensitized macrophages increase their NOS activity following a second rhTNF treatment. In these experiments, NADPH diaphorase histochemical staining intensity was measured due to its direct proportionality to NOS activity. In macrophage-ENS co-cultures, NOS activity levels in macrophages do not significantly differ between S/S controls and cultures exposed to a single treatment of rhTNF (either T/S or S/t treatments). However, in co-cultures exposed to rhTNF 1 day previously, macrophages display significantly increased NOS activity in response to further administration of rhTNF (T/t treatment). NOS activity was evaluated 1 day following administration of the second dose of rhTNF. Increasing grayscale values indicate decreased NADPH diaphorase staining intensity. *$p<0.01$ (one-way ANOVA followed by post-hoc Tukey/Kramer analysis).

and colon (Fig. 36), while HO-1 up-regulation occurred only in colon. Ileal cultures of enteric neurons also responded to rhTNF by up-regulation of Il-1α expression but not that of HO-1, as predicted from *in vivo* findings.

Macrophage mediation of rhTNF-induced neurodegeneration

Repeated exposure of enteric cell cultures to rhTNF (T/t treatment) failed to induce neuronal cell death. Co-culture of enteric neurons together with the RAW 264.7 murine macrophage cell line (at an approximately 2:5 macrophage: neuron ratio) did not in itself induce neuronal cell death, nor did single exposure of these co-cultures to rhTNF (T/S or S/t treatments). However, when ENS/macrophage co-cultures were exposed to rhTNF under sensitized conditions (T/t treatment) nearly 50% of all neurons died within 24 hrs of the second administration of rhTNF (Fig.37).

NOS activity of rhTNF-sensitized macrophages

The NADPH diaphorase staining intensity (indicative of NOS activity) of macrophages co-cultured with enteric neurons did not differ from saline controls (S/S) in cultures treated with a single dose of rhTNF (either T/S or S/t treatment). However, under sensitized conditions (T/t treatment), NOS activity within macrophages increased significantly, as demonstrated by the marked decrease in NADPH diaphorase grayscale values (Fig. 38).

Regional Specificity of rhTNF-α Self-Sensitization within Murine Intestine

	Duodenum	Jejenum	Ileum	Colon
HO-1	No change	No change	No change	sensitization
IL-1α	No change	No change	sensitization	sensitization
TUNEL	No change	No change	sensitization	No change
c-fos	No change	No change	sensitization	No change
Thymidylate synthase	No change	sensitization	sensitization	No change

Table 3. Regional specificity of rhTNF self-sensitization effects within the murine intestine. All observed intestinal responses to rhTNF injection were observed only upon prior sensitization to rhTNF (T/t treatment). Differences in cell sensitivity within different intestinal regions (duodenum, jejenum, ileum and colon) to rhTNF exposure were observed. The majority of detectable intestinal responses were localized to the ileum and colon, with the duodenum and jejenum demonstrating a relative insensitivity to rhTNF.

Discussion

This study tested the hypothesis that elevated concentrations of rhTNF induce neurodegeneration within the ENS. Although we found evidence that enteric neurons were stimulated by cytotoxic concentrations of rhTNF both *in vivo* and *in vitro*, no neurodegeneration occurred in either of these experimental systems. The experimental hypothesis was then modified to test whether rhTNF interaction with inflammatory macrophages was capable of inducing degeneration within enteric neurons, which was proven to be the case by *in vitro* experimentation. Whereas in the absence of macrophages, rhTNF induced functional alterations within enteric neurons without any accompanying toxicity, in the presence of macrophages, these same concentrations of rhTNF induced marked degeneration within enteric neurons. By extrapolation, the failure of rhTNF to induce neuronal cell death *in vivo* is suggested to be due to the lack of inflammatory macrophage infiltration within neuromuscular layers.

Our observed induction of IL-1α expression in enteric neurons by rhTNF (both *in vivo* and *in vitro*) corresponds with the known ability of rhTNF to induce IL-1 production within a variety of cell types (Dinarello et al., 1986; Hoffmann, 1986; Hurst and Collins, 1993). Indeed, TNF has been shown to suppress norepinephrine release from the rat longitudinal muscle-myenteric plexus preparation, in part via the synthesis and release of interleukin-1 (Hurst and Collins, 1994). In the *in vivo* ENS, excessive production of IL-1 has been proposed to induce alterations in myenteric nerve function and lead to motility alterations characteristic of IBD (Jacobson et al., 1997). Our finding of the selective induction of IL-1α but not IL-1β by rhTNF was unexpected, as the biological activities of these cytokines are reported to be indistinguishable (Dinarello, 1997). One explanation of

our results is that IL-1α may simply be preferentially expressed by enteric neurons. If this is true, then this may represent a unique pharmacological means of targeting these cells. We are currently exploring this notion.

Previous studies have found that TNF induces up-regulation of HO-1 expression, which can protect against intracellular TNF-induced oxidative stress (Terry et al., 1998; 1999; Petrache et al., 2000). On this basis, we propose that our findings of HO-1 induction within enteric neurons following T/t treatment represent an anti-apoptotic response to rhTNF actions. It is noteworthy that this effect was seen within the colon but not within the ileum.

In vitro studies were used to test our hypothesis that macrophages mediate rhTNF-induced degeneration of enteric neurons. In these studies, enteric neurodegeneration occurred only in response to T/t treatment, exclusively within ENS-macrophage co-cultures. These observations correlate with previous *in vitro* studies that have noted macrophage-induced degeneration of sympathetic neurons (Arantes et al., 2000). The mechanism(s) by which macrophages mediate this rhTNF-induced enteric neurodegeneration are currently under study in our laboratory. Significant increases in NOS activity were noted in macrophages exposed to *in vitro* T/t treatment, indicating rhTNF activation of these cells. Whether such increases in NO production are involved in the neurotoxic actions of macrophages is not known. However, macrophages activated by rhTNF may release neurotoxic compound(s) that can act directly or synergistically with other compounds to induce neuronal cell death. Substances released from enteric neurons in response to either rhTNF or to macrophage-derived factors (eg. ROS), may also modulate or contribute to cytotoxic processes. Indeed, NO released from

macrophages may react with neuronal ROS produced in response to TNF (Larrick and Wright, 1990) to produce compounds with high toxicity for enteric neurons.

In our experiments we found no immunohistochemically detectable changes within the murine intestine in response to an initial exposure of a low dose of rhTNF. However, upon injection of a second dose *one-fourth* that of the initial dose, rhTNF provoked marked intestinal responses. These experimental findings demonstrate that in the intestine, rhTNF sensitizes both enteric neurons and intestinal mucosa to the effects of further rhTNF exposures. By these means, otherwise sub-effective doses of rhTNF induce marked responses within intestinal cells. These findings of rhTNF self-sensitization within murine intestine correspond with our previous characterization of this phenomenon within the CNS (Hayley et al., 1999).

The biological processes underlying rhTNF self-sensitization remain unknown. Within this self-sensitization phenomenon, initial rhTNF doses have been suggested to induce effects similar to those of transcriptional inhibitors, which increase cell sensitivity to the cytotoxic effects of TNF-receptor interaction by preventing expression of cellular protective factors. By these means, rhTNF may sensitize cells to the toxic effects of subsequent rhTNF administration by removing endogenous cell death inhibitors or endogenous protective factors (Sipe et al., 1996; Venters et al., 2000). Alternatively, synergistic interaction of TNF with other endogenous cytokines on a given cell may underlie this process. In this scenario, initial administration of rhTNF would enhance synthesis of a cytokine such as IFN-γ, which is known to have direct synergistic action with TNF (Bundschuh et al., 1997; Nansen et al., 1997). IFN-γ could then synergize with rhTNF injected at a later time point to induce potentiated cellular responses.

Within our *in vivo* and *in vitro* experiments, sensitized cell responses to otherwise sub-effective doses of rhTNF were highly similar to those induced by single high doses of rhTNF; indicating that sensitization does not alter the biological targets and physiological processes of TNF. That these sensitized responses also resembled specific characteristics of intestinal pathogenesis suggests a possible role for TNF self-sensitization processes within disease pathogenesis. Our experimental T/t treatment induced crypt cell proliferation consistent both with the known ability of inflammatory TNF concentrations to induce crypt cell proliferation, and with the crypt cell hyperplasia typical of IBD (Ruemmele et al., 1998; Dionne et al., 1998; Bradley et al., 1997; Mizoguchi et al., 1997; MacDonald et al., 2000). Similarly, apoptosis induced in the villus epithelial cells by T/t treatment resembles both the response to murine TNF-p55 interaction in mice (Piguet et al., 1998), and the severe epithelial cell loss typical of IBD (Dieleman et al., 1996). TNF is also known to induce synthesis of many proteins including the transcription factor c-fos (Saklatavala, 1995; Larrick and Wright, 1990). We observed induction of c-fos expression following rhTNF injection within stem cells of the intestinal crypts, indicative of activation or injury of these cells. Interestingly, our observation that rhTNF induced expression of the transcription factor c-fos 1 hr following a second exposure to rhTNF, corresponds with the known ability of TNF to induce c-fos expression 20-90 minutes after cell excitation (Morgan et al., 1987; Larrick and Wright, 1990; Saklatavala, 1995). Finally, the villi epithelial cell apoptosis induced by TNF self-sensitization mimics both the severe goblet cell loss and epithelial cell sloughing characteristic of IBD pathogenesis, and the actions of elevated levels of TNF on these

cells (Murch et al., 1993). Neurons demonstrate sensitization to rhTNF both *in vivo* and *in vitro*; suggesting that sensitization processes are intrinsic to cells.

The TNF self-sensitization observed in intestinal cells exhibited a time-dependency of action; maximal sensitization was evident at 1 and 7 day exposure intervals, though undetectable by 28 day intervals. A similar sensitization profile is seen for brain monoamine level changes in response to rhTNF treatment (Hayley et al., 1999). TNF is the first cytokine for which such a temporal pattern of sensitization has been identified. We propose that this slow desensitization of tissue responses to rhTNF with time likely represents a gradual breakdown of factors involved in the sensitization process.

In this study, intestinal cells also displayed regional variations in their responses to rhTNF exposure. *In vivo*, rhTNF actions were localized predominantly to ileal and colonic regions, which are those intestinal regions that are the primary sites of IBD pathogenesis. These findings suggest that TNF, which plays a fundamental role in IBD pathogenesis, may help determine IBD localization to these intestinal regions. The correspondence of the *in vivo* and the *in vitro* actions of rhTNF on neuronal function demonstrate that the regional variations in rhTNF actions represent some fundamental difference between the ENS of ileal and colonic regions.

In conclusion, in the absence of any overt inflammation rhTNF rapidly induces cell stress responses and damage in many cell types throughout intestinal layers, including enteric neurons. However, there is an absolute requirement for macrophages to be present in order for rhTNF to be capable of inducing enteric neurodegeneration. The mechanisms by which macrophages mediate this rhTNF neurotoxicity are currently

unknown. Thus, we have shown rhTNF to exert both macrophage-dependent and macrophage-independent actions within enteric neurons and intestinal tissues. We thus hypothesize that within IBD, TNF-α also damages intestinal tissues both by itself and by interaction with infiltrating inflammatory cells.

rhTNF is capable of sensitizing tissue to the effects of further exposure to this cytokine. However, tissue sensitivity to rhTNF varies between intestinal regions, and rhTNF actions are subject to a time-dependent self-sensitization effect. TNF is the first cytokine in which such temporal patterns of self-sensitization have been characterized. This may provide a new perspective with respect to its actions in IBD pathogenesis as well as for other disease treatments that target this cytokine. For example, knowledge of the temporal pattern of TNF sensitization in the intestine could be used to design more efficacious dosing regimens for the administration of anti-TNF antibodies in the treatment of IBD.

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Chapter 4. DISCUSSION

Although ENS cell loss and alteration of cell function are characteristic of intestinal inflammation and disease, little is known of the underlying neuropathogenesis. Clinical symptoms of enteric nerve damage include disordered or impaired gastrointestinal movement, nausea, vomiting, weight loss and malnutrition (Oehmichen and Reifferscheid, 1977). Although a causal relationship between intestinal inflammation and enteric neurodegeneration has been established (Sanovic et al., 1999), little is known of the specific inflammatory mediators involved in such damage.

The ENS differs markedly from the other nervous systems of the body, the CNS and the ANS, at several different levels. At an organizational level, the ENS does not exhibit the regional specializations of the CNS; instead ganglia are composed of multiple neuronal subtypes with mixed functions (Eckblad et al., 1988). Neurochemically, all identified CNS neurotransmitters have also been found within the ENS, however they do not always provoke the same functional responses (Krantis et al., 1980; Krantis et al., 1998). Functionally, the ENS also differs from the CNS and PNS; exhibiting considerable plasticity in its ability to continually remodel neural connections (Collins, 1996; Giaroni et al., 1999). Additionally, the blood-ganglia of the ENS, intended to protect enteric neurons, is considerably more permeable to toxins than the restrictive blood-brain and blood-nerve barriers (Kiernan, 1996; Allen and Kiernan, 1990; 1994; Goldstein and Betz, 1986). Thus, the ENS represents a unique nervous system of the body. Despite considerable understanding of neurotoxic processes in the CNS, the multiple differences between the CNS and ENS necessitates specific investigation of neurotoxic processes within the ENS. The similarities noted between the susceptibility of

ENS and CNS neurons to damage, and the suggestion of common intracellular damage mechanisms for these two systems were an unexpected finding of our studies, given the unique properties of these two systems. These results suggest that despite variability in the organizational structure and specific functional properties of individual neuronal populations, that neuronal susceptibility to toxic insults and intracellular damage mechanisms may represent fundamental properties common to neurons in general.

The primary goal of this research project was to determine the role of specific inflammatory mediators in enteric neurodegeneration. Standard protocols do not exist for examination of toxicity and degeneration within the ENS. Thus, the initial goal of this research project was the development of an experimental system for characterization and quantitative evaluation of ENS responses to toxic insult. We used the well-characterized inflammatory neurodegenerative processes within the CNS as a model on which to base our studies of ENS neurodegeneration. A major outcome of this research was our development of a versatile and physiologically relevant *in vitro* system optimized for the study of toxicity within the adult ENS (Chapter 2). Such ENS cultures represent an ideal *in vitro* system in which to study neurodegenerative and neuroprotective processes specific to the adult ENS.

A common, although widely unrecognized limitation of cell culture systems is their unsuitability for toxicity studies. Many cellular substrates required to produce viable cell cultures rapidly degrade as a consequence of cellular insult, effectively preventing observation of cell culture response to insult. Thus, a feature of our culture system was the selection of a cell culture substrate that would both maximize cell viability in culture and resist degradation upon cellular insult. Our culture system

represents one of the few culture systems specifically designed for neurotoxicological experimentation that has been extensively characterized and whose physiological relevance to the *in vivo* adult ENS has been demonstrated.

The question as to why adult enteric neurons, as opposed to other peripheral neurons or neurons from the CNS, survive the dissociation process and remain viable in long term culture, remains unanswered. The high plasticity of enteric neurons into maturity may be an underlying factor in their ability to survive cell culture. Whereas fetal neuronal populations are commonly subject to highly variable surroundings, the environment of adult neurons is generally quite stable. Enteric neurons, however, retain high plasticity into maturity, as they are subject to a continually changing microenvironment in which muscular contractions regularly distort the intestine and in which constant sloughing of villi epithelia necessitates the constant re-establishment of neural connections (Giaroni et al., 1999). This plasticity of adult enteric neurons may provide them with the ability to withstand dissociation stresses and to rapidly adapt to the cell culture environment.

The study of isolated neurons is hampered by the need to use the isolated cells immediately, which often requires a very large concentration of resources, and in many instances it is not possible to undertake multiple evaluation of these cells. The ability to utilize freshly harvested cells and store aliquoted 'sister cells' for subsequent evaluation affords the researcher with considerable flexibility in timing studies. Our development of a cryopreservation storage procedure of dissociated ENS cells for culture that are morphologically, neurochemically, and electrophysiologically identical to freshly plated cultures, and in which no significant loss of cellular viability occurs, greatly facilitates

the use of these cultures for experimentation (Chapter 2). These cryopreserved cells are readily available for use as required, and are easily transported for use in collaborating laboratories. Additional advantages of such cryopreservation processes include the access of laboratories to ENS cell cultures without their requirement for animal care facilities, costly specialized dissection equipment, and specially trained personnel. Although previous articles have detailed the cryopreservation storage of viable neural tissues and grafts (Petite and Calvet, 1997; Frodl et al., 1995) there are no previously published reports of efficient cryopreservation of dissociated neurons. Our development of this cryopreservation procedure represents the first known case of the cryopreservation storage of dissociated adult enteric neurons for subsequent culture.

An unexpected characteristic of our cryopreserved enteric neurons was their superior quality when cultured compared to freshly plated neurons. Typically, cryopreserved neurons displayed a profusion of extended processes at the 24 hr time point as compared to only occasional processes extended by freshly-dissociated neuronal cultures. We propose that some feature of the cryoprotectant permeabilizes cell walls, accelerating neuronal interactions with the culture environment and conferring an advantage to cell sustainability. This phenomenon needs to be investigated since determination of the mechanisms underlying the accelerated plating ability of cryopreserved neurons may help optimize cell plating and viability in other cell culture systems. Indeed, our protocol for the cryopreservation storage of enteric neurons has recently been adapted and optimized for the cryopreservation storage of several populations of dissociated fetal CNS neurons. These cryopreserved enteric and CNS neurons are currently being developed as marketable scientific tools intended to save

laboratories the considerable time and expense of developing their own primary neuronal cultures for experimental use.

The results of our neurotoxicological assays showed the neurodegenerative abilities of the ROS hydrogen peroxide and NO, and of the processes of oxidative stress and hypoxia/anoxia in the cultured ENS. Our findings constituted the first evidence that ROS and anoxia-reoxygenation act directly on the ENS to induce enteric neurodegeneration. These studies also contributed to the functional knowledge of ENS pathogenesis.

Within these studies, some common characteristics of ENS degeneration were noted. Our studies showed that for varied toxic insults, neurons were markedly more susceptible to damage and degeneration than were associated glial cells. Such high sensitivity to insult may represent a general property of enteric neurons, similar to that of CNS neurons under such conditions (Goldberg et al., 1987; Regan and Choi, 1991). Neurons are thought to be highly sensitive to insult due to their high dependency on constant energy supplies and specific pH levels and ion concentrations for maintenance of normal cellular functions and cell viability (Sapolsky, 1992). We found that inflammatory mediators induce primarily apoptotic cell death and this corresponds to the findings of Kirchgessner et al. (1997) using guinea pig ENS. To date, apoptosis in the ENS has generally been disregarded as an important cell death mechanism in disease pathogenesis. However, identification of cell death mechanisms in the ENS is important for the development of therapeutic treatments for gut disease that address specific cell death mechanisms. Thus, as these findings suggest the prevalence of apoptosis in ENS

degeneration, it is critical that future studies must focus on the role of this cell death mechanism in ENS disease pathogenesis.

The clinical significance of ROS, oxidative stress and anoxia/reoxygenation in inducing ENS degeneration *in vitro* remains to be determined. Although these inflammatory mediators have known cytotoxicity in intestinal tissues during inflammatory bowel diseases (Otamiri and Sjodahl, 1991) at concentrations toxic to ENS neurons *in vitro*, it remains to be determined if neurons act as the biological targets of these agents *in vivo*. *In vivo* studies need to establish if neurodegeneration occurs at those toxin concentrations present during disease pathogenesis and if any cellular interactions occur that may modulate the effect of toxins on neuronal survival.

Within these initial studies of ENS neurotoxicity, several similarities have been noted between the susceptibility of enteric neurons and CNS neurons to damage in vitro. As there is, in general, a paucity of data regarding ENS damage processes, our studies were, to a high degree, modeled upon CNS toxicity studies. We suggest that such CNS studies represent a relevant model upon which to base our investigations of ENS neurodegeneration. We plan to take advantage of the relative wealth of knowledge of CNS neuropathogenesis for use as a basis for our continued study of ENS neurodegenerative processes and their underlying mechanisms.

Within our studies, the enteric neuroprotective ability of the neurotrophic factor GDNF was investigated. GDNF provided significant protection against neuronal damage in our cultures. However, this neuroprotective ability was limited to neurodegeneration induced by hydrogen peroxide insult. We postulate that this selective neuroprotective ability may be due to GDNF's counterposition with only specific apoptotic pathways, as

found in other neuronal systems (Sawada et al., 2000). GDNF represents the first reported neuroprotective factor in the adult ENS *in vitro*. Further studies are required to establish GDNF's actions *in vivo*, and to determine a possible therapeutic role for this molecule. Our discovery of GDNF as a neuroprotective molecule in the adult ENS suggests that other members of the GDNF family of neurotrophic factors may also have similar neuroprotective properties. One such candidate molecule that merits investigation is neurturin, a potent survival factor for several populations of central and peripheral neurons that is also found at high concentrations in the adult intestine (Golden et al., 1999).

Our *in vivo* experiments in Chapter 3 are based upon the recently described phenomenon that the cytokine rhTNF can sensitize CNS tissues and animal illness behavior to the effects of further TNF exposures in a time-dependent manner (Hayley et al., 1999). We found that rhTNF sensitizes intestinal tissues to the effects of further administrations of rhTNF; sensitization decreasing with increasing intervals between rhTNF exposures. TNF is the first cytokine for which such a time-dependent self-sensitization has been identified. This sensitization phenomenon is likely to occur physiologically and may have important repercussions for analysis of TNF actions within disease pathogenesis in that the effects of TNF cannot be determined by simple measurement of its concentration within tissues, but must rather be evaluated in the context of its recent tissue concentration history. Indeed, tissue sensitization to the effects of TNF over a given time period may underlie previous findings that IBD patients have increased occurrence of irritable bowel syndrome (IBS) and that in animal models, previous inflammation increases colonic responses to stress (Collins et al., 1996; 1999).

A possible role for such time-dependent TNF sensitization must be considered for its implications for inflammatory disease pathogenesis and for the administration of anti-TNF antibody therapies, as are in clinical trial use for IBD pathogenesis (van Deventer and Camoglio, 1997).

Our experiments determined that, within a given animal, the temporal patterns of TNF sensitization varied between tissues. Additionally, those temporal sensitization patterns for generalized illness behaviors were markedly different from those for the intestinal effects. Previously, sequence modification of this cytokine has separated the biological functions and systemic toxicity of TNF (Novakovic et al., 1997). Our current findings indicate that these TNF functions are inherently dissociable by means of their differing temporal sensitization profiles. This means of dissociation may have potential application to the use of TNF as an anti-tumor agent, as it use has been traditionally hampered by the attendant cytotoxicity which accompanies its anti-tumor efficacy (Abbruzzese et al., 1990). TNF's time-dependent sensitization of tissues to its further administration represents a additional level of functional complexity for this cytokine that requires further study to elucidate the mechanism by which this occurs.

TNF sensitization within tissues also has considerable practical importance. With sensitization, the amount of TNF required to produce a given effect within tissues is considerably lessened. For example, by means of sensitization, a dose $1/100^{th}$ that normally required was used to induce a 50% morbidity in mice (unpublished observations). Thus, with the use of TNF sensitization, *in vivo* rhTNF experimentation that is otherwise cost prohibitive can be performed at a fraction of the normal cost. By

these means, we readily extended our *in vitro* experimental studies of enteric neurodegeneration to *in vivo* models.

In our experiments, we used *in vitro* cultures as complementary systems for the rapid resolution of experimental anomalies that presented during *in vivo* experimentation and analysis. A marked accordance of *in vivo* and *in vitro* data was observed, validating the use of such dual systems. The use of correlating *in vivo* and *in vitro* experimental systems permit a wide range of questions to be rapidly investigated. We suggest that this bipartite method of experimentation act as a versatile and comprehensive system for examination of enteric neuropathogenesis. Future studies are planned to develop a murine ENS culture system similar to that of the rat ENS. With *in vivo* cytokine studies and gene knockout experiments performed in mice, a comprehensive experimental system employing *in vivo* and *in vitro* models from the same species would be extremely advantageous.

Within our studies, rhTNF was shown to induce IL-1 expression within enteric neurons of the murine intestine in the absence of overt inflammation. Previous experiments have noted that in an experimental animal model of colitis, myenteric nerve function was altered at both inflamed and non-inflamed sites via an IL-1-mediated process that did not require T lymphocytes (Jacobson et al., 1997). We suggest TNF as a candidate molecule for the induction of such IL-1 mediated alterations of ENS function. Interestingly, ultrastructural changes in enteric neurons (Dvorak et al., 1980) and intestinal transit and motor changes (Rao et al., 1987; Manousos and Salem, 1965) have been noted within non-inflamed regions of intestine during clinical IBD pathology. These functional and structural changes of non-inflamed regions are thought to suggest

some unknown systemic component of the inflammatory process (Jacobson et al., 1995). We suggest that during IBD pathogenesis, pro-inflammatory cytokine concentrations may be elevated in regions beyond the extent of overt inflammation. The demonstrated actions of pro-inflammatory cytokines on the ENS, independent of inflammatory cell involvement, may participate in the ENS alterations within non-inflamed regions observed clinically in IBD patients.

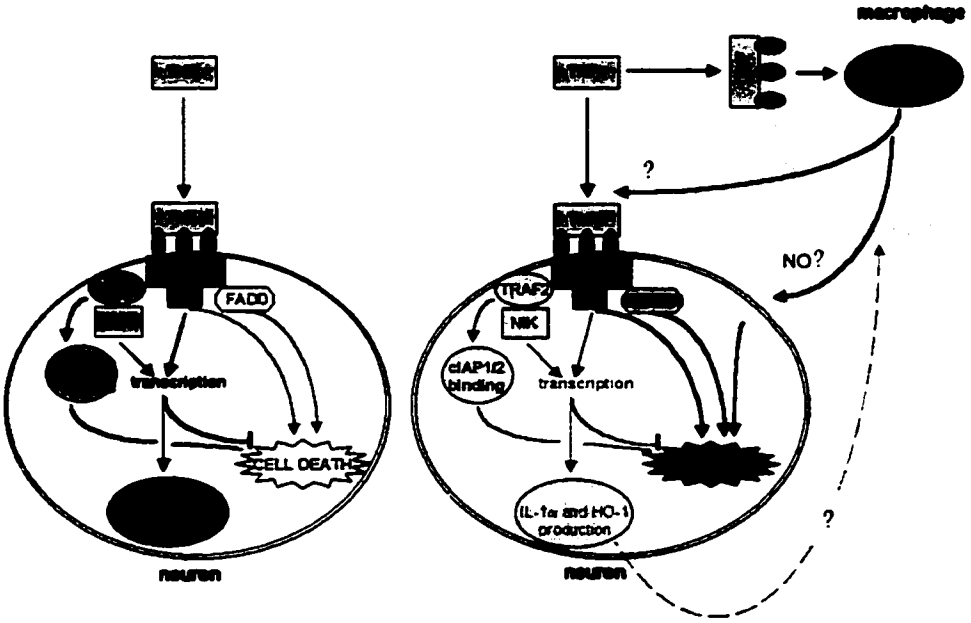
Our results showed that while TNF itself could alter neuronal function, activated macrophages were required to mediate TNF-induced degeneration of enteric neurons. These findings cast the notion of the clinical prevention of ENS neurodegeneration in inflammatory bowel diseases in a new light. We have determined that although TNF itself induces many of the intestinal changes characteristic of IBD, irreparable ENS damage occurs only upon infiltration of inflammatory macrophages. Furthermore, these findings illustrate that mechanisms underlying induction of functional stress within enteric neurons are not necessarily those mechanisms that induce degeneration of these neurons. This separation of altered neuronal function vs. neurodegeneration has not been addressed in previous studies. In particular, whether the degree of insult determines one or other, or both of these outcomes.

We also determined that during ENS degeneration, activated macrophages displayed increased levels of NOS activity; implicating NO as a neurotoxic agent released by these cells. These findings correspond with our previous studies that demonstrated NO, either alone or in combination with other ROS (that may be produced by direct TNF actions on neurons), can be neurotoxic in the ENS. Further studies will be

directed towards direct determination of the role that these molecules play in the macrophage-mediated degeneration of neurons induced by TNF.

Although the mechanism by which TNF and macrophages interact to induce enteric neurodegeneration is not known, several possibilities are suggested (Figure 39). Macrophage activation may be induced directly by TNF interaction with TNF receptors on macrophages themselves. Alternatively, TNF actions on neurons may induce these cells to produce some factor which, when released, activates macrophages. Once activated, macrophages may produce some factor that is directly toxic for neurons, or which indirectly induces neurodegeneration. Scenarios for indirect neurotoxicity include the reaction of factors released by macrophages with TNF itself or with other factors released from neurons to produce neurotoxic compounds.

Within these studies, we determined that a wide variety of insults, including oxidative stress, lipid peroxidation, anoxia-reoxygenation and inflammatory cytokine actions could induce enteric neurodegeneration *in vitro*. As stated previously, it remains to be determined whether such insults induce enteric neurodegeneration *in vivo*. However, the fact that enteric neurodegeneration is a feature of a wide variety of both intestinal and primarily extra-intestinal diseases suggests that amongst these diseases, a number of different damage mechanisms may be involved in ENS degeneration. Indeed, the variations noted between ENS damage characteristics of such diseases (as noted within Table 2, Chapter 1), corresponds with this notion. Within our studies, we noted that the resistance of NOS neurons to HNE-induced neurodegeneration, and remarked on its similarity to the selective survival of NOS neurons characteristic of certain intestinal diseases (Chapter 3). During future studies of ENS neurodegeneration, correlation of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transcription
transcription
CELL DEATH
IL-1α and HO-1 production
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Figure 39. Schematic of proposed mechanisms whereby macrophages mediate rhTNF-induced neurotoxicity. In the absence of macrophages, rhTNF likely interacts directly with TNF receptors on enteric neurons to induce gene transcription, resulting in IL-1α up-regulation and HO-1 expression. In the presence of macrophages, neuronal cell death occurs in response to several possible mechanisms of action. rhTNF activation of macrophages may induce these cells to produce some factor (such as NO) that induces neuronal cell death either directly or in combination with rhTNF or other molecules produced as a result or rhTNF actions. Factors produced by neurons in response to rhTNF could themselves activate macrophages, while rhTNF and/or macrophage actions on neurons could also cause the release of factors which themselves modulate the cytotoxic processes involved in enteric neurodegeneration.

ENS damage characteristics, including those neuronal subtypes preferentially damaged or spared, with known ENS damage characteristics of specific diseases may identify candidate molecules involved in clinical ENS damage.

In summary, this thesis research has demonstrated direct actions of specific inflammatory mediators and inflammatory processes on the ENS that culminate in the degeneration of enteric neurons *in vitro*. This work contributes to the sparse data available on neurodegenerative agents in the ENS, and implicates specific inflammatory mediators in such actions. These studies reveal the existence of both macrophage-mediated and macrophage-independent neurotoxic processes within the ENS, provide some insight into the methodological characteristics of ENS degeneration, and identify a neuroprotective agent of potential therapeutic use. Our development of a versatile and physiologically relevant experimental system for such studies is expected to be highly advantageous for further investigation into inflammatory degeneration of enteric neurons.

Chapter 5. LITERATURE CITED

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