SELECTIVE DAMAGE TO AND PROTECTION OF ENTERIC NEURONS IN VITRO IN MODELS OF ISCHEMIA AND REPERFUSION

by

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Abstract

The dysregulation of motility typical in inflammatory bowel disease (IBD) indicates involvement of the enteric nervous system (ENS). Indeed, animal models of IBD have demonstrated that intestinal inflammation is typified by the early loss of enteric neurons. It has been proposed that ischemia may occur in intestinal inflammation, due to the organization of splanchnic circulation combined with local inflammatory damage. Therefore, we hypothesized that the ENS experiences ischemic challenge in inflammation, which we investigated by probing for hypoxia-inducible factor 1-α (HIF1-α) in the 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-model of colitis in adult rats. By Day 2 of TNBS-induced colitis, HIF1-α presence in the nuclei of myenteric neurons had significantly increased, indicating hypoxic challenge.

Despite the potential for ischemia to occur in inflammation, little is known about the impact of the resultant metabolic inhibition on enteric neurons. We further hypothesized that metabolic challenge would cause neuronal damage. Glucose deprivation, hypoxia and treatment with an electron transport chain (ETC) uncoupler caused neuron loss and axonal damage in in vitro co-cultures of myenteric neurons, intestinal smooth muscle cells and glia. Furthermore, we investigated the potential impact of subsequent reperfusion on myenteric neurons by resupplying glucose following a period of deprivation. Resupply of glucose was observed to cause acute neuron loss in vitro.

Lastly, if the ENS is damaged by ischemia and ischemia-reperfusion, it is then important to investigate methods of neuroprotection. In the CNS, glial cell line-derived neurotrophic factor (GDNF) and HIF1-α have both been demonstrated to be neuroprotective during metabolic inhibition. In co-cultures, GDNF was observed to prevent neuron loss by an ETC uncoupler or glucose deprivation. Hypoxic preconditioning also protected against neuron damage in a HIF1-α
dependent manner. We concluded that the ENS is vulnerable to ischemia and ischemia-reperfusion occurring in inflammation. Further, GDNF and HIF1-α can be neuroprotective during metabolic challenge.
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<tr>
<td>CD</td>
<td>Crohn’s disease</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
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<tr>
<td>DMOG</td>
<td>dimethyloxalylglycine</td>
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<tr>
<td>DNBS</td>
<td>dinitrobenzene sulfonic acid</td>
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<tr>
<td>DNP</td>
<td>2,4-dinitrophenol</td>
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<td>ENS</td>
<td>enteric nervous system</td>
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<tr>
<td>ETC</td>
<td>electron transport chain</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>GDNF</td>
<td>glial cell-line derived neurotrophic factor</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GFRα1</td>
<td>GDNF family receptor alpha 1</td>
</tr>
<tr>
<td>Hanks’</td>
<td>Hanks’ balanced salt solution</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid</td>
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<td>HIF1-α</td>
<td>hypoxia inducible factor 1-alpha</td>
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<td>IBD</td>
<td>inflammatory bowel disease</td>
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<tr>
<td>IBS</td>
<td>irritable bowel syndrome</td>
</tr>
<tr>
<td>ICC</td>
<td>interstititial cells of Cajal</td>
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<td>IEC-6</td>
<td>intestinal epithelial cell line 6</td>
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<td>IL-1</td>
<td>interleukin 1</td>
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<td>ISMC</td>
<td>intestinal smooth muscle cell</td>
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<td>MCAO</td>
<td>middle cerebral artery occlusion</td>
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<td>NBF</td>
<td>neutral buffered formalin</td>
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<td>NGF</td>
<td>nerve growth factor</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<td>NOS</td>
<td>nitric oxide synthase</td>
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<td>NT-3</td>
<td>neurotrophin 3</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>OMG</td>
<td>3-O-Methyl-D-glucopyranose</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>SM/MP</td>
<td>smooth muscle and myenteric plexus layers</td>
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<td>SNAP-25</td>
<td>synaptosome-associate protein 25 kD</td>
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<td>TNBS</td>
<td>2, 4, 6-trinitrobenzene sulfonic acid</td>
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<tr>
<td>TNF-α</td>
<td>tumour necrosis factor alpha</td>
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<td>UC</td>
<td>ulcerative colitis</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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Chapter 1

Introduction

Chronic inflammation of the intestine can be debilitating, leading to abdominal pain, both diarrhea and constipation, anemia, and weight loss. As of 2012, inflammatory bowel disease (IBD) affects over 230 000 Canadians and costs Canada over $2.8 billion annually, in both direct and indirect costs (Rocchi et al, 2012). Currently, treatment of IBD focuses on resolution of symptoms during ‘flares’ and maintenance of remission, employing both biological and surgical interventions, with a large variation in success. Greater understanding of how inflammation damages and changes the intestine will lead to more successful therapies.

1.1 Neuron loss in intestinal inflammation

The enteric nervous system (ENS), often described as the ‘brain of the gut’, works independently of the brain and spinal cord. The ENS works with intestinal smooth muscle cells (ISMCs) and the interstitial cells of Cajal (ICC) to coordinate movement of the intestine, including persistaltic and churning motions. Enteric neurons are arranged in two layers of ganglia within the gut wall. The submucosal (Meissner’s) plexus is located within the submucosa and the myenteric (Auerbach’s) plexus is located between the circular and longitudinal muscle layers (Fig. 1A). Axons extend from neurons to innervate surrounding ISMCs. A healthy ENS is required for proper digestion and absorption of food and clearance of waste. Damage to the ENS can cause dysregulation of contractile activity and loss of coordination, potentially leading to malabsorption of nutrients and water and constipation or diarrhea. The dysregulation of motility typical in IBD indicates involvement of the ENS.
Indeed, animal models of IBD have demonstrated that intestinal inflammation is typified by two early events: loss of enteric neurons and proliferation of ISMCs (Sanovic et al., 1999). In the dinitrobenzene sulfonic acid (DNBS)-induced colitis model in rats, significant neuronal loss is observed in the inflamed region within 24 hours (Sanovic et al., 1999; Figure 1B). By days 4 to 6, when inflammation had begun to subside, only 49% of neurons remained. Similarly, in whole mount tissues in mice, DNBS-induced colitis caused a 60% decrease in the number of myenteric neurons (Boyer et al., 2005). In 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in guinea pigs, about 20% of myenteric neurons were lost within 12 hr of TNBS-administration (Linden et al., 2005). These changes in the ENS are an early and sensitive indicator of damage and can themselves have functional consequences.

Similar results have been observed in tissue obtained from patients with IBD. Several groups have reported structural abnormalities in the ENS in IBD, including loss of neurons and axonal damage (Steinhoff et al., 1988; Dvorak et al., 1993). Though this has not been firmly established, neuron loss may be a permanent change to the intestine caused by inflammation. Rapid growth and extension of axons has been observed in the DNBS-induced colitis model as an intrinsic method of compensation following neuron loss (Sanovic et al., 1999; LourensSEN et al., 2005). However, normal function and motility of the smooth muscle layers may not be fully restored in all cases. Even in non-chronic inflammation, the loss of neurons may cause post-inflammatory conditions, including motility disorders or irritable bowel syndrome (IBS), although this is still unproven. Therefore, further understanding is necessary to prevent inflammatory damage to the ENS, which may alleviate some post-inflammatory symptoms.
Fig. 1. A. Schematic representation of a transverse section through the intestine. The enteric nervous system is organized into two plexii: the submucosal plexus is within the submucosa and the myenteric plexus is between the circular and longitudinal smooth muscle layers. (altered from Smout et al., 1992). B. Neurons are lost early in chemically-induced colitis. Almost 50% of neurons are lost by D4 TNBS-induced colitis. (altered from Sanovic et al., 1999).
1.2 Ischemia in intestinal inflammation as a cause of neuronal damage

Though acute inflammation is commonly associated with hyperemia in other areas of the body, it has been proposed that intestinal inflammation may involve both hyperemia and ischemia. Indeed, the impaired healing of ulcerations in the gut mucosa in IBD has similarities with leg ulcers seen in ischemic conditions in peripheral vascular disease (Hatoum et al., 2005). Ischemia is insufficient blood supply to meet the metabolic needs of an area. Ischemia may be due to reduced blood supply, such as in occlusions or vasoconstriction, or due to local increased metabolic demands. In most cases, ischemia then leads to both insufficient glucose availability and hypoxia. Considering that neurons of the central nervous system (CNS) are known to be particularly vulnerable to ischemia, ischemia in the intestine may contribute to the deleterious effects of inflammation on the ENS.

1.2.1 Previous evidence and possible causes of ischemia in inflammation

The organization of splanchnic circulation combined with local inflammatory damage may mean that the distribution of blood flow in the intestine becomes inadequate in inflammation. In Crohn’s disease (CD), atrophy of the media of the submucosal arteries, which leads to increased vascular resistance, has been observed even in the early stages of the disease (Funayama et al., 1999). Moreover, higher amounts of platelet aggregates were found in venous samples from CD patients compared to arterial samples or controls, indicating that thrombosis may be occurring in the mesenteric microvasculature in CD (Collins CE et al., 1997). Mucosal biopsies from ulcerative colitis (UC) patients also contain intravascular platelet aggregates (Collins CE et al., 1997). Other factors that have been linked to causing ischemia in intestinal inflammation include an increase in local vasoconstrictor products such as the endothelins and
thromboxane, a decrease in local nitric oxide (NO) production, and decreased responsiveness to NO (Hatoum et al., 2005). More recently, angiogenesis has been shown to be upregulated in IBD, perhaps in response to the factors already discussed (Deban et al., 2008). However, this response appears to be maladaptive, since it leads to anastomoses that further impair even distribution of blood flow throughout the gut wall, while concurrently perpetuating inflammatory conditions (Deban et al., 2008). Therefore, intestinal inflammation may consist of both ischemia and hyperemia, occurring simultaneously in different locations or alternating at the same location.

However, even in normal conditions, the gut mucosa is naturally predisposed to ischemia. Due to the nature of the cross-current exchange of gases up into the villi, there is less of a concentration gradient to force oxygen into the mucosal tissue (Tao et al., 1995). This natural hypoxia may be worsened in inflammation, which may be partly responsible for the damaging effects of inflammation on the mucosa. However, the question of interest is whether the deeper smooth muscle and myenteric plexus (SM/MP) layers experience ischemia and hypoxia in inflammation.

1.2.2 Methods of visualizing hypoxia

The studies discussed above used diverse techniques to study the possibility of ischemia in IBD, including angiographic studies. Nevertheless, in order to confirm what areas of the gut wall actually experience ischemia in inflammation, the aim would be to find a histochemical marker of ischemia. Since hypoxia is a major result of ischemia, markers of hypoxia can also be used to visualize ischemia. Previous studies have identified hypoxia in intestinal inflammation in the mucosa. Yet, all of these studies have relied on the use of a single marker, Hypoxyprobe™, a
patented molecule purported to directly detect low oxygen levels in tissue. In the dextran sodium sulfate-induced model of UC in rats, Hypoxyprobe™ indicated hypoxia in the mucosa (Tolstanova et al., 2012). Analogous results were also seen in the TNBS model in mice (Karhausen et al., 2014). Though these studies of animal models of IBD did not indicate hypoxia in the SM/MP layers of the intestinal wall, hypoxia has been visualized using Hypoxyprobe™ in enteric neurons during occlusive ischemia (Fisher et al., 2013). This indicates that, if ischemia were to occur in intestinal inflammation, deeper neuromuscular layers may become affected. Nevertheless, the Hypoxyprobe™ method does not indicate physiological response or challenge, beyond stating that Hypoxyprobe™ stains where hypoxia is expected. Therefore, an alternative marker for hypoxia is necessary, which may be used as an indicator of ischemia.

Hypoxia-inducible factor 1-α (HIF1-α) is a transcription factor involved in metabolic regulation, known to be involved in the cell’s response to hypoxia. Constantly produced by cells, oxygen is required for HIF1-α’s breakdown. Consequently, in low oxygen conditions, HIF1-α is free to translocate to the nucleus to act as a transcription factor (Sharp & Bernaudin, 2004). Some of HIF1-α’s gene targets work to increase oxygen delivery, such as vascular endothelial growth factor (VEGF). However, HIF1-α’s main function is to decrease oxygen demand by downregulating mitochondrial metabolism and upregulating glycolysis (Bergeron et al., 2000; Papandreau et al., 2006). Where there is ischemia, hypoxia is a natural outcome. Where there is increased HIF1-α activity, and no other cause of hypoxia is present, ischemia is a likely possibility. Indeed, HIF1-α has been shown to be upregulated in cerebral ischemia (Jin et al., 2000; Dharap et al., 2009). Thus, by probing for HIF1-α in TNBS-colitis, areas of the intestinal wall experiencing hypoxia can be identified, as a method of evaluating ischemic challenge.
1.2.3 Consequences of ischemia on the ENS

Despite the potential for ischemia to occur in inflammation and reach the SM/MP layers, little research has been done on the impact of metabolic inhibition on enteric neurons. In the CNS, neurons are reliant on glucose metabolism for energy, largely because of the reduced lactate availability due to the blood-brain barrier (Boumezbeur et al., 2010). Furthermore, neurons are known to have a high energy demand compared to many other cell types, including muscle, potentially due to the need to fire action potentials (Ames, 2000). Thus, glucose and oxygen deprivation is known to cause neuronal damage in the CNS. Complete oxygen deprivation, such as in asphyxiation, will cause brain damage after only 6 minutes, while partial glucose or oxygen deprivation, such as in an occlusion, will cause brain damage within an hour (Kaplan et al., 1991). Other tissue can withstand these conditions for longer periods of time before cell death.

Based on these findings in the CNS, enteric neurons may be particularly vulnerable to metabolic inhibition compared to neighbouring smooth muscle cells. Though the ENS does not have a structure similar to the blood-brain barrier (Kiernan, 1996), enteric neurons may have an inherent higher energy need similar to neurons of the CNS. Moreover, enteric neurons have been observed to be selectively susceptible to other challenges, including oxidative stress. In in vitro cultures of rat SM/MP, 24 hr exposure to 75 μM H$_2$O$_2$ caused a 50% decrease in neuron survival, while smooth muscle cells remained unaffected (Lourenssen et al., 2009). In the same way, metabolic inhibition may be particularly damaging to the ENS.
1.2.4 *In vitro* models of ischemia

There are several available models that could be used to examine the impact of ischemia on enteric neurons *in vitro*, which would mimic the metabolic challenge that would be experienced in ischemia. Among these, 2,4-dinitrophenol (DNP) has been used to inhibit oxidative phosphorylation. By shuttling protons across the mitochondrial membrane, DNP uncouples the electron transport chain (ETC). Sold over the Internet as a weight loss agent, DNP has also been used extensively in research as a model of ischemia, particularly in cardiology, and has even been referred to as “chemical hypoxia” (Murata *et al.*, 2001). DNP does leave the glycolytic pathway intact, preventing complete energy deprivation. Interestingly, DNP has been shown to be neurotoxic to rat hippocampal neurons (Dubinsky & Rothman, 1991), while rat cortical astrocytes are unaffected in similar conditions (Swanson, 1992). Again, this suggests that neurons, including enteric neurons, may be more susceptible to metabolic inhibition than other cell types.

Secondly, glucose deprivation, as would be expected in ischemia, can be achieved by competitive inhibition of glucose usage by 3-O-methyl-D-glucopyranose (OMG). OMG is a non-metabolizable analogue to glucose. By changing the ratios of glucose:OMG, the level of metabolic inhibition can be altered, while still maintaining molar balance in media. As mentioned before, the brain is susceptible to glucose deprivation, partly due to the blood-brain barrier preventing access to lactate. However, in glucose deprivation, astrocytes in the brain will produce and shuttle lactate to neurons, so that CNS neurons do use lactate in limited amounts (Magistretti, 2006; Bergersen, 2007). It is not yet known whether enteric neurons have comparable access and use of lactate as CNS neurons. Therefore, the impact of glucose deprivation on enteric neurons is not fully understood.
Lastly, oxygen deprivation would also be the natural consequence of ischemia. Using a hypoxic chamber, the amount of oxygen available to cells can be controlled. Hypoxia, separate from other consequences of ischemia and inflammation, is known to cause neuronal apoptosis in the CNS (Banasiak & Haddad, 1998). In cultures of mouse cerebellum, exposure to 2% O₂ for one hour led to dendritic degeneration and disorganization of mitochondrial cristae, while astrocytes and oligodendrocytes remained unaffected (Kim, 1975). Several factors in hypoxia induced neuron death have been identified. Energy deprivation, in itself, is not the primary cause of death, but rather a rise in intracellular calcium and creation of free radicals (Goldberg & Choi, 1993; Banasiak et al., 2000). Nonetheless, despite the large amount of research into hypoxia in the CNS, hypoxia in the ENS has not been widely studied. In summary, DNP, OMG substitution of glucose and hypoxia can be used in vitro to study the impact ischemia may have on enteric neurons.

1.3 Further consequences of ischemia-reperfusion injury

Whenever there is the danger of ischemia, there is also the potential for ischemia-reperfusion injury. Generally, ischemia-reperfusion injury occurs when the lack of blood supply or oxygen for a period creates conditions where restoration of blood supply results in inflammation and oxidative damage, rather than a return to normal function (Dorweiler et al., 2007). Ischemia-reperfusion injury began to be recognized and described in the early 1970s. The occurrence was first systematically studied in rat hindleg, but has been observed and studied in tissues and organs throughout the body. (Dorweiler et al., 2007). It has been demonstrated that the recruitment of neutrophils and the release of proinflammatory cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α), are critical events in ischemia-reperfusion
injury (Jean et al., 1998). In the CNS, not only does ischemia cause neuronal injury in the CNS, reperfusion following ischemia can often cause a greater degree of damage, which is often seen in strokes. Though ischemia, perhaps due to an occlusion, may last below a critical time period so that only a small area of the brain is affected, restoration of blood flow often causes an acute extension of the infarct area (Jean et al., 1998). In rat brains, the infarct volume after 24 hours of middle cerebral artery occlusion was 28% of the infarct volume of 2 hours of occlusion followed by 24 hours of reperfusion (Aronowski et al., 1997). Moreover, after a 10 min bilateral carotid artery occlusion in rats, restoration of blood flow in hyperoxic conditions reduced hippocampal neuron survival compared to restoration of blood flow in normoxic conditions (Hazelton et al., 2010). Accordingly, similar to neurons of the CNS, enteric neurons may also be susceptible to ischemia-reperfusion injury.

In the context of the gut, ischemia-reperfusion injury is of particular concern in surgery, where blood flow may be clamped off for brief periods of time. However, considering the remitting and relapsing nature of IBD, if ischemia occurs in inflammation, then so shall ischemia-reperfusion. Ischemia-reperfusion injury has been studied in the intestine, but has focused on the impact on the mucosa. Overall, ischemia-reperfusion causes an increased mucosal permeability due to weakened tight junctions and direct damage to epithelial cells by free radicals (Kong et al., 1998). In monolayers of epithelial cell lines, Caco-2 and intestinal epithelial cell line 6 (IEC-6), the transepithelial electrical resistance does not decrease after an hour of hypoxia, but does significantly decrease after an hour of reoxygenation (Xu et al., 1998). Moreover, bacterial translocation of E.coli is not seen in hypoxia alone, but is seen after reoxygenation (Xu et al., 1998). The importance of ischemia-reperfusion events to the intestine
has been recognized, but the effect of them on the deeper SM/MP layers is still only beginning (Pontell et al., 2011).

1.4 Methods of preventing injury from ischemia and reperfusion

If ischemia does occur in inflammation and is experienced by the deeper SM/MP layers, the next goal is to find methods to prevent ischemic damage and ischemia-reperfusion injury to the ENS. There are two main strategies one can adopt: 1) mitigate ischemia in general; or 2) enable cells to survive the ischemic and reperfusion challenge. This project will demonstrate neuron loss in metabolic challenge in vitro and then investigate methods of preventing that damage.

1.4.1 The role of GDNF in neuron survival and axon outgrowth

Neurotrophins are critical factors that govern embryonic development and subsequent plasticity of neural systems. Yet, the role of neurotrophins in the post-natal intestine has not been fully identified. Recently, several advancements have been made in understanding the role of glial cell line-derived neurotrophic factor (GDNF) post-natally. GDNF’s primary developmental role is to drive the migration, proliferation and differentiation of the enteric neural crest-derived cells (Pichel et al., 1996). Loss of GDNF signaling results in hypoganglionosis or aganglionosis of the distal colon (Uesaka & Enomoto, 2010). It should be noted that, despite its name, GDNF is produced by intestinal smooth muscle cells, rather than enteric glia (Rodrigues et al., 2011, Peters et al., 1998). The production of GDNF by smooth muscle cells continues after development is complete. GDNF promotes neuronal survival and axonal outgrowth of neurons in the post-natal ENS (Rodrigues et al., 2011). Furthermore, inflammatory factors have been
observed to stimulate the expression of GDNF from ISMCs *in vitro*, increasing axonal outgrowth among those target cells (Gougeon *et al.*, 2013). GDNF may be able to have similar neuroprotective effects in intestinal ischemia.

Indeed, GDNF has repeatedly and consistently been neuroprotective in focal and global ischemia in the brain (Duarte *et al.*, 2012). In the adult rat brain, exogenous GDNF applied intraventricularly prior to a transient middle cerebral artery occlusion (tMCAO) increased reparative proliferation of new neurons by 91% of control (Dempsey *et al.*, 2003). Similarly, exogenous GDNF applied to the cortical surface of rat brain following tMCAO was found to prevent apoptosis and autophagy of neurons, effectively decreasing infarct size (Shang *et al.*, 2010). The prior implantation of stem cells modified to overexpress GDNF was also protective in tMCAO (Chen *et al.*, 2009). Considering the positive effect GDNF has on the ENS in other forms of challenge and on the CNS in ischemic challenge, it should be investigated whether GDNF can prevent neuron loss in ischemia and ischemia-reperfusion in the intestine.

1.4.2 Preconditioning as an inducer of HIF1-α

Since the early 1990s, hypoxic preconditioning has been investigated for its ability to prevent injury from future hypoxia and metabolic inhibition. By exposing tissue to sub-acute hypoxia for a short period of time, cellular adaptations take place that then prepare the tissue for the greater subsequent hypoxic challenge or ischemia. Rat hearts exposed to hypoxia for 5 min and allowed to recover for an hour before being reexposed to hypoxia for 30 min regained 95% of function, while hearts that had not been preconditioned with hypoxia regained only 64% of function (Lasley *et al.*, 1993). Correspondingly, transplantation of mesenchymal stem cells cultured in 0.5% O₂ into rat heart during myocardial infarction encouraged restorative
angiogenesis and improved function in comparison to transplantation of stem cells cultured in normoxia (Hu et al., 2008). Similar results are seen in the CNS. In one study, rat pups exposed for 3 hours to 8% O₂ 24 hours prior to having the left cerebral artery occluded had no change in hemispheric weight, while non-preconditioned rats had a 30% decrease in hemispheric weight (Gidday et al., 1994). Thus, it is established that preconditioning does protect against hypoxia and ischemia.

In application, it would be difficult to precondition tissue in expectation of ischemic insult. Instead, understanding the cellular adaptations undergone during preconditioning may provide methods of equipping cells for ischemia. Investigation into the mechanism behind preconditioning has implicated induction of HIF1-α as a key intracellular modification that enables ischemic tolerance (Bergeron et al., 2000). Studies have typically made this conclusion based on the observation that HIF1-α is upregulated and stabilized during hypoxic preconditioning and that inhibition of the transcriptional targets of HIF1-α prevents the protective effects of preconditioning (Chavez et al., 2000; Liu et al., 2005; Liu & Alkayed, 2005). One study has artificially stabilized HIF1-α by inhibiting prolyl hydroxylases that would normally break HIF1-α down, which resulted in protection of the mucosa in TNBS-colitis (Robinson et al., 2008). Though preconditioning may not be a viable treatment option, manipulating HIF1-α and downstream targets during ischemic challenge may be beneficial.

Interestingly, though induction of HIF1-α has largely been linked to beneficial protective effects in ischemia and other metabolic challenge, there have been a few studies that suggest HIF1-α may have a negative effect in subsequent reperfusion. Partially HIF1-α deficient mice were found to have greater mucosal injury compared to control mice after superior mesenteric artery occlusion, but had less mucosal damage compared to control after subsequent reperfusion
(Kannan et al., 2010). Thus, HIF1-α activity may be adaptive proximally in ischemia, but then becomes a maladaptive long-term response.

1.5 Conclusions, experimental goals and hypotheses

Therefore, as this discussion indicates, further investigation into the possibility of ischemia in intestinal inflammation is vital. Rather than use Hypoxylene™, probing for HIF1-α will provide greater insight into what is actually being experienced by the cells. If ischemia and hypoxia is present in the myenteric plexus during inflammation, it may be partly responsible for the early loss of neurons that is observed in colitis. Studying neuron survival in vitro in metabolic inhibition and models of ischemia will provide insight as to how neurons react to ischemia, especially in contrast to the response from adjacent cells. If neurons are damaged by metabolic inhibition, methods of neuroprotection must be considered. Both GDNF and preconditioning have proven to be effective in protecting neurons of the CNS in ischemia. After considering the effects of ischemia, the impact of ensuing reperfusion on the ENS must also be studied. Importantly, the responses of neurons to ischemia and to reperfusion may vary greatly. This may mean that strategies for coping with ischemia may become maladaptive upon reperfusion.

Overall, this project provides further insight into the mechanism of the neurotoxic effects of inflammation on the ENS. As well, the examination of ischemia-reperfusion injury may have implications for intestinal surgery. Approaches for protecting the ENS in these circumstances may help alleviate the disrupted motility that is seen in IBD.
Based on the previous research discussed, the underlying hypothesis of this project is that ischemia occurs in inflammation and damages the ENS. The following project goals were formed:

- Develop *in vitro* models of ischemia and reperfusion in the SM/MP layers of the rat intestine
- Probe for the expression of HIF1-α in TNBS-colitis, which is suggestive of ischemia occurring in intestinal inflammation
- Explore the impact of ischemia and metabolic inhibition on the ENS
- Explore the further effects of ischemia-reperfusion on the ENS
- Investigate methods of protecting the ENS in ischemia and ischemia-reperfusion, including the ability of GDNF and HIF1-α to be protective
Chapter 2

Materials and Methods

2.1 Animals

Sprague-Dawley rats were obtained from Charles River Laboratories (Montreal, PQ, Canada) and housed in pairs in microfilter-isolated cages, with free access to food and water. Primary cell cultures and whole mounts were derived from neonatal rats bred in the GIDRU animal facility. Chemical colitis was induced in adult male rats. All procedures received prior approval by the Queen’s University Animal Care Committee and conformed to the Canadian Council of Animal Care guidelines.

2.2 TNBS-Induced Colitis

TNBS was used as a chemically-induced colitis model, as described previously (Stanzel et al., 2008). Adult male Sprague-Dawley rats were lightly anaesthetized with inhaled isoflurane. 500 μL of 200 mM TNBS (Sigma, St. Louis, Missouri, USA) dissolved in 50% ethanol was then instilled rectally into the colon, 8 cm proximal to the anus, using a PE-50 catheter. Animals were sacrificed at the 12 hr, 1 day and 2 day time points and tissue from the mid-descending colon was removed and fixed in 4% neutral buffered formalin (NBF), followed by routine processing and paraffin embedding. Sections were also obtained from rats receiving no treatment and from rats receiving only ethanol vehicle control.
2.3 Isolation of Primary Co-cultures

Co-cultures of neonatal rat myenteric neurons, smooth muscle cells and glia were generated from neonatal Sprague-Dawley rats (2-8 days of age), as previously described (Gougeon et al., 2013). The small intestine was isolated. In HEPES-buffered Hank’s saline (pH 7.35) (H/H), the mesentery completely removed and the mucosa and muscularis mucosa was separated from the SM/MP layers and discarded. The SM/MP was then incubated in 0.25% trypsin II (Sigma) in H/H for 75 min. The tissue was resuspended in Dulbecco’s Modified Eagle Medium (DMEM; GIBCO, Carlsbad, California, USA) containing 10% fetal calf serum (FCS) and trituturated to yield a cell suspension. Cell number was determined using a hemocytometer and then cells were plated in 24-well plates on collagen-coated glass cover slips at a density ranging from 3.8-4.5 x 10^5 cells/mL in DMEM with a final concentration of 5% FCS. After 48 hr incubation, the cultures were serum-deprived for an additional 24 hr before exposure to test conditions. A schematic of the co-culture protocol is displayed in Fig. 2.

2.4 Whole Mount Tissue Preparation

Whole mount preparations were also obtained from post-natal rats (11-17 days of age), as an ex vivo model. The SM/MP was removed from the small intestine and 0.5 cm long segments were incubated in DMEM in test conditions in 1 mL of media in 24-well plates.

2.5 Treatment of Tissue

The following treatments were applied to co-cultures and/or whole mounts:

- DNP (0.75 mM; Fluka, St. Louis, Missouri, USA) was applied for indicated times.
OMG (25 mM; Santa Cruz, Dallas, Texas, USA) was dissolved in no glucose DMEM (GIBCO, Carlsbad, California, USA), and mixed with full-glucose DMEM to desired ratio of glucose:OMG, which was then applied for indicated times.

To mimic hypoxia, tissue was placed in a chamber set to 1% or 5% O₂ as specified (C-Chamber, with ProOx P110; BioSpherix, Lacona, New York, USA) for indicated times, where O₂ was replaced with N₂. When moving from hypoxia to normoxia and vice versa, co-cultures were place in pre-quilibrated media.

GDNF (50 ng/mL; Peprotech, Rocky Hill, New Jersey, USA) was applied 8 hr prior to and/or simultaneously with other treatments.

Chetomin (10 nM; Tocris, Bristol, United Kingdom) was applied 30 min prior to other treatments, and remained present in solution throughout treatment.

CoCl₂ (100-300 μM; Sigma, St. Louis, Missouri, USA) was applied for 4 hr prior to other treatment, and removed at that time.

2.6 Transfection of Co-cultures

Co-cultures were transfected with a plasmid expressing green fluorescent protein (GFP)-GDNF (1 μg/mL) using lipofectamine (Invitrogen, Carlsbad, California, USA), according to manufacturer’s protocol, 36-48 hr prior to treatment with metabolic inhibitors. 24 hr after initial set-up, the 5% FCS media was replaced with antibiotic-free, serum-free DMEM. 1 μg/mL of plasmid was mixed with antibiotic-free, serum-free DMEM, enough for 250 uL/well, for 5 min at room temperature. Simultaneously, 1 μL/well lipofectamine was mixed with antibody-free, serum-free DMEM, enough for 250 uL/well, for 5 min. The plasmid and lipofectamine was then mixed together for 20 min at room temperature,
Cell Dissociation

5% FCS
48 hr

0% FCS
24 hr

varied

Fix, stain, count

B

C

D

E

F

G
Fig. 2. Co-culture protocol. A. Schematic representation of co-culture protocol. Myenteric neurons, glia and intestinal smooth muscles obtained from the SM/MP of the small intestine of neonatal rats were cultured in 5% FCS. After 48 hr, the co-cultures were serum deprived for an additional 24 hr before exposure to test conditions. After fixation, immunohistochemical analysis for changes in neurons and axons was performed. B-D. Immunohistochemical labelling of control co-culture with HuD (B), SNAP-25 (C), and Hoechst (D). E. Colour composite of B, C and D. Scale bars, 25 µm. F-G. Counting method in co-cultures. All neurons in every third field of view in horizontal and vertical axes were counted (F). Axonal intersections with the horizontal and vertical midlines were counted (G).
before being distributed to wells (500 uL/well) and allowed to incubate for 6 hr. 5% FCS DMEM was then replaced. GFP expression was visually assessed prior to treatment.

2.7 Immunocytochemistry and Immunohistochemistry

After treatment in test conditions, co-cultures were fixed for 10 min in NBF, followed by three 10 minute washes in PBS. The cultures were then exposed to mouse anti-HuD (1:1000; Molecular Probes, Eugene, Oregon, USA) and rabbit anti-SNAP-25 (1:5000; Sigma, St. Louis, Missouri, USA) in PBS containing 0.2% Tween (PBS-T) at 4°C overnight. After removal of primary antibodies, culture wells were washed with PBS three times, 10 minutes each, and then exposed to Alexa Fluor secondary antibodies: goat anti-rabbit Alexa-555 (1:4000; Molecular Probes, Eugene, Oregon, USA) and goat anti-mouse Alexa-488 (1:2000; Molecular Probes, Eugene, Oregon, USA) in 0.2% PBS-T for 1 hr at room temperature. Hoechst 333258 (0.1 µg/mL; Sigma, St. Louis, Missouri, USA) was used to label nuclei. In some cases, co-cultures were probed for HIF1-α expression using rabbit anti-HIF1-α (1:200; 479SS, Novus, Littleton, Colorado, USA), along with HuD. Labelled coverslips were then mounted onto slides and preserved at 4°C.

After treatment in test conditions, whole mounts were pinned out to a standard area, fixed for 1 hr in 4% NBF and then washed in PBS for 5 min. Whole mounts were then exposed to mouse anti-HuD (1:1000) and rabbit anti-glial fibrillary acidic protein (GFAP) (1:250; Biomedical Technologies, Ward Hill, Massachusetts, USA) in PBS-T at 4°C overnight. Whole mounts were then exposed to Alexa Fluor secondary antibodies: goat anti-rabbit Alexa-555 (1:1000) and goat anti-mouse (1:1000) in 0.2% PBS-T for 2 hr at room temperature and Hoechst 333258 (0.1 µg/mL; Sigma, St. Louis, Missouri, USA) was used to label nuclei.
4 μm sections were dried either overnight or for 1 hr at 37°C and were then rehydrated. Sections were then treated with target retrieval solution according to manufacturer’s protocol (Dako, Glostrup, Denmark), and labeled with mouse anti-Hud and rabbit anti-HiF1-α overnight at room temperature in a humidity chamber. Following a 1 hr exposure to secondary antibodies in a humidity chamber, sections were treated with Hoechst.

All tissue was visualized using an Olympus microscope (BX51 or 60) and imaged using Image Pro Plus (Media Cybernetics).

2.8 Quantification of Neurons and Axons

In co-cultures, neuron and axon numbers were quantified as described earlier using an Olympus microscope (BX60) (Gougeon et al., 2013). Neuron numbers were assessed by counting HuD-positive neurons in every third field in a horizontal and vertical strip, representing 2.8% of the total surface area of the coverslip. To quantify axon numbers, consecutive fields were examined in a horizontal and vertical strip across each coverslip, and the number of SNAP-25-immunoreactive axons intersecting the midline of the microscopic field was counted. Axonal density was calculated as the square of axon number from above, divided by the neuron number from above. Duplicates were made of every condition and averaged together.

In whole mounts, neuronal density was measured by counting the number of HuD-positive neurons present in defined lengths of tissue. The neuronal density was then expressed as neurons/mm. Since D11-D14 neonatal rats were used, enteric neurons were present in long uninterrupted parallel chains, and not yet in grouped together into discrete ganglia, which is seen in adult intestine. In each whole mount, 3 non-adjacent fields were assessed. Within each field, three separate lengths of neurons were measured and counted. Duplicates were made from every
animal for every condition and averaged together. The total area assessed for each animal for every condition was 586 mm².

2.9 Statistical Analysis

Data analysis was performed using GraphPad Prism software. All values are expressed as the average ± the standard error of the mean of n animals. The statistical significance was assumed for p≤0.05 using two-way or one-way repeated measures analysis of variance (ANOVA) with Bonferroni or Dunnett’s post-tests as appropriate, to evaluate differences between multiple populations, or two-tailed paired Student’s t-test, to compare differences between two populations.
Chapter 3

Results

3.1 HIF1-α expression is upregulated in chronic intestinal inflammation in vivo

To investigate whether the SM/MP layers experience hypoxic challenge in intestinal inflammation, sections from the colon of rats with TNBS-induced colitis were labeled with antibodies to HIF1-α and compared to expression in control tissue. In control sections, less HIF1-α was visible in the SM/MP layers and, when present, was found mainly in the cytoplasm of cells (Fig. 3A,C). In control sections, HIF1-α was apparent at the tips of villi in the mucosa, but not in the deeper SM/MP layers. In D1 TNBS sections, the amount of HIF1-α was visibly increased in the SM/MP layers and was present in both the nuclei and the cytoplasm of cells, both neurons and surrounding smooth muscle cells (Fig. 3B,D). When quantified, the percentage of neurons that contained HIF1-α in the nucleus in control sections was 22% ± 3.7%, while in D1 TNBS 68% ± 9.0% (n=3, p<0.05) of neurons were HIF1-α positive in the nucleus (Fig. 3E). The increased nuclear presence of HIF1-α in neurons in inflammation was evidence of ischemia in the SM/MP layers.

3.2 Neurotoxic effects of metabolic inhibition

The increased nuclear translocation of HIF1-α in the SM/MP layers in TNBS-colitis indicates that the ENS is experiencing and responding to metabolic challenge and hypoxia. Therefore, in vitro models of ischemia were used to assess the impact of metabolic challenge on enteric neurons and surrounding cells. The models used were: reduced glucose, DNP, and hypoxia.
Fig. 3. Nuclear translocation of HIF-1α was evidence for ischemia in myenteric plexus neurons during colitis. Immunocytochemistry for HIF-1α was performed on sections of colon from control adult male rats or during TNBS-induced colitis. A-B. Representative composite images of HIF1-α expression (red) and Hoechst staining (blue) in control (A) and D2 TNBS (B) in SM/MP layers of a rat colon cross-section. C-D. Corresponding magnified representative composite images of HIF1-α (red) and HuD expression (green) in control (C) and D2 TNBS (D) rat colon myenteric plexus demonstrated stabilization and nuclear translocation of HIF1-α in inflammation in the myenteric plexus and surrounding smooth muscle. Scale bars, 25 µm. E. Quantification of the percentage of neurons with nuclear HIF1-α, demonstrating the upregulation of HIF1-α in inflammation (n=3; *P<0.05; Student’s t-test)
3.2.1 Reduced glucose availability causes selective neuron loss in vitro

Primary co-cultures of neonatal rat intestine, consisting of smooth muscle cells, neurons and glia, were created by enzymatic dissociation of the SM/MP layers. In control co-cultures, neurons were abundant and typically found in groups of 3 or more neurons. Axons were long, unbroken and highly branching, creating an extensive network among neurons, as well as connecting to surrounding accessory cells (Fig. 4A). In control co-cultures derived from neonatal D2-8 rats, there were typically 7336 ± 393.6 neurons/coverglass (n=44), comprising 4.9 ± 0.2% of the total cell population.

Initial experiments tested the effects of glucose deprivation on enteric neurons, as might occur in ischemia. Glucose availability was altered by changing the ratio of glucose:OMG, a non-metabolizable glucose analogue, which maintained molar balance. As glucose decreased and OMG increased, neuron survival significantly and proportionately decreased (Fig. 4B,C). For example, when 80% of the glucose was replaced with OMG, neuron number was reduced to 67 ± 4% (n=3, P<0.01) of control (Fig. 4C). Additionally, while complete glucose deprivation for 16 hr did not cause neuron loss (Fig. 8B,D), after 24 hr significant neuron loss (55 ± 13%; n=3, P<0.01) had occurred (Fig. 4C). Axonal density also decreased and remaining axons were visibly damaged, appearing broken in multiple places (Fig. 4B,D). Furthermore, whether the neuron damage was specific to a neuron type was tested by staining for neuronal nitric oxide synthase (nNOS). In control neonatal co-cultures, nitrergic neurons were 29.2 ± 1.9% (n=3) of total neuron population. In reduced glucose conditions, nitrergic neurons were 30.8 ± 3.3% (n=3, p>0.05) of total neuron population. Thus, it was determined that reduction of glucose does not affect nitrergic neurons to a greater or lesser extent than cholinergic neurons.
Fig. 4. Glucose deprivation caused selective and proportional neuron loss and decreased axonal density in vitro. The concentration of available glucose in neonatal rat co-cultures was altered by substitution with OMG for 24 hr, prior to immunocytochemical assessment. A-B. Representative composite images of neuronal cell bodies expressing HuD (green), pan-neuronal marker SNAP-25 (red) and all nuclei stained with Hoechst (blue) in in vitro co-cultures after 24 hr treatment with full glucose control (A) and after 80% reduction of glucose concentration (B). Control co-cultures had large axonal networks, connecting groups of multiple neurons, while glucose deprivation caused loss of neurons and shortened and damaged axons. Arrows point to breaks in axons. Scale bars, 25 µm. C-D. Neuron and total cell number (C) and axonal density (D) relative to control in vitro after 24 hr in media containing specified concentration of glucose, demonstrating selective progressive neuron loss in glucose deprivation (n=3; *P<0.01 vs control; two-way repeated measures ANOVA with Bonferroni post-test) and loss of total axonal density in glucose deprivation (n=3; *P<0.05 vs control; one-way ANOVA).
To determine if reduced glucose affected all cell types present in co-cultures, total cell number was assessed by counting Hoechst labelled nuclei. Compared to control, the number of total cells was not significantly altered in reduced glucose conditions. Glia were assessed by counting the number of GFAP-immunopositive cells. In control neonatal co-cultures, glia formed 33.1 ± 0.2% (n=3) of the total cell population. In reduced glucose conditions, glia comprised 30.8 ±3.3% (n=3, p>0.05) of the total cell population. Thus, glia were not significantly affected by reduction of glucose.

Therefore, reduced glucose availability caused selective myenteric neuron loss in vitro. This indicated the vulnerability of enteric neurons to metabolic challenge, as might be seen in ischemia in inflammation.

3.2.2 Disruption of the electron transport chain causes selective neuron loss in vitro

DNP, which uncouples the ETC, was also used as a form of metabolic inhibition. Co-cultures treated for 24 hr in concentrations up to 0.5 mM DNP showed no significant change in neuron number or morphology. However effects were apparent after 24 hr treatment in media containing concentrations of DNP above 0.5 mM.

Overall, DNP caused similar outcomes in primary co-cultures as reduced glucose, including significant neuron loss and axonal damage (Fig. 5B). After 24 hr treatment with 0.75 mM DNP, neuron survival in primary cultures was reduced to 73 ± 6% (n=11, p<0.01) of control (Fig. 5C). Neuron damage was not specific to any subtype, as nitrergic neurons in DNP-treated co-cultures composed 27.3 ± 2.0% (n=3, p>0.01) of the total neuron population, similar to control.
Fig. 5. Uncoupling the electron transport chain with DNP caused selective neuron loss in vitro. A-B. Representative composite images of neuron bodies expressing HuD (green), pan-neuronal marker SNAP-25 (red) and all nuclei stained with Hoechst (blue) in a control co-culture (A) and after 24 hr treatment with 0.75 mM DNP (B). After treatment with DNP, neurons were lost and axons were damaged and shortened. Scale bars, 25 µm. C. Neuron and total cell number relative to control in vitro after treatment with 0.75 mM DNP for specified length of time, demonstrating selective progressive neuron damage by DNP (n=4-11; *P<0.01 vs control; two-Way ANOVA with Bonferroni post-test)
Total cell number was not significantly affected, indicating that neuron loss was a selective outcome in the co-cultures (Fig. 5C). Further, the specific analysis of glial cells revealed that DNP had no effect on glia. In DNP-treated co-cultures, glia comprised 32.2 ± 1.3% (n=3, p>0.05) of total cells, which was similar to control.

The neurotoxic effects of DNP were progressive with increased lengths of time or concentration. For example, after 48 hr of treatment with 0.75 mM DNP, neuron number was reduced to 46 ± 4% (n=4, p<0.05) of control and the total cell number was not significantly changed from control (Fig 5C). However, if the concentration of DNP was increased to 1 mM, non-selective toxicity was observed, as total cell number was reduced to 73 ± 7% (n=9) of control after 48 hr. Thus, disruption of the ETC, which is a possible effect of ischemia, also caused selective neuron loss in vitro. This was further evidence of the vulnerability of the ENS to metabolic inhibition.

3.2.3 Hypoxia causes early neuron loss in vitro

A hypoxic chamber was used to examine the effects of O$_2$ deprivation on enteric neurons, as may be encountered in ischemia. Primary co-cultures were exposed to 1% O$_2$ for various amounts of time, followed by assessment of neuron and total cell number. After exposure to 1% O$_2$ for 4 hr, neuron survival was decreased and, though axonal density was not yet reduced, axons on remaining neurons were visibly damaged (Fig 6B). In co-cultures exposed to 1% O$_2$ for 4 hr, neuron number was reduced to 72 ± 9% (n=4, p<0.05) of control (Fig. 6C). However, while hypoxia for 4 hr showed selective toxicity, exposure for 24 hr resulted in non-selective toxicity. Thus, neurons demonstrated a more rapid response to hypoxia than surrounding cells. Furthermore, neuron death did not progress further after 4 hr, despite continuing hypoxia.
Fig. 6. Hypoxia caused early myenteric neuron loss in vitro. A-B. Representative composite images of neuron bodies expressing HuD (green), pan-neuronal marker SNAP-25 (red) and all nuclei stained with Hoechst (blue) in a control co-culture (A) and after 4 hr exposure to 1% O\(_2\) in a hypoxic chamber. In hypoxia, neuron survival was decreased and axons were extensively damaged, while surrounding accessory cells were unaffected. Scale bars, 25 µm. C. Neuron and total cell number relative to time-matched control in vitro after exposure to 1% O\(_2\) for indicated time, demonstrating earlier loss of neurons compared to accessory cells (n=4; *P<0.05 vs control; two-way ANOVA with Bonferroni post-test).
3.2.4 Metabolic inhibition causes neural damage *ex vivo*

The three models of metabolic inhibition were also tested *ex vivo* whole mounts of the SM/MP layers of the neonatal rat intestine. After treatment and fixation, the whole mounts were stained for both HuD and GFAP, to identify neuron bodies and glial elements respectively. In whole mounts from neonatal animals, neurons were typically localized in chain-like structures, with no easily identifiable individual ganglia. In control whole mounts, the chains appeared unbroken, 2-3 neurons wide, and the glial elements were seen surrounding the neurons like an interconnected lattice (Fig. 7A,C). Neuron density was quantified by counting the number of neurons per length in a given chain, and was typically 102.5 ± 3.9 (n=9) neurons/mm. This appearance was altered after exposure to metabolic inhibition, where the chain-like structures appeared broken and were typically only 1-2 neurons wide. Furthermore, GFAP-positive extensions surrounded the HuD-immunonegative regions, appearing as holes or gaps within the lattice network of glia, suggesting loss of previous neuronal structure (Fig 7B,D). When quantified, in whole mounts exposed to an 80% reduction in glucose concentration, neuron density was significantly reduced to 64.1 ± 4.0 (n=4, p<0.01) of control. Similar results are seen in exposure to DNP and hypoxia (Fig 7E). Thus, all three models of metabolic inhibition used caused damage to the myenteric neurons *ex vivo*, while the appearance of glia remained unchanged. These results further confirmed the findings of the *in vitro* experiments, that enteric neurons were especially vulnerable to metabolic inhibition and conditions similar to ischemia.
Fig. 7. Metabolic challenge with glucose deprivation, DNP, or hypoxia caused neuron loss in *ex vivo* whole mounts. A-B. Representative images of neuron bodies expressing HuD in whole mounts of SM/MP layers of juvenile rat colon after 24 hr treatment with full glucose control (A) and 80% reduction of glucose concentration (B). C-D. Representative composite images of neuron bodies expressing HuD (green) and glial cells expressing GFAP (red) in SM/MP whole mounts in control (C) and 80% reduced glucose (D). In control conditions, neurons were present in unbroken chains, surrounded by glial cells. In reduced glucose conditions, neurons were lost, while glial cells were unaffected, creating holes in the chain appearance (arrow). Scale bars, 25 µm. E. Neuronal density (neurons/mm) in whole mounts after 24 hr treatment with specified conditions (n=3-6; *P<0.01 vs control; two-tailed Mann-Whitney test).
3.3 Ischemia-reperfusion injury to the ENS

Reperfusion has often been observed to cause greater damage than the originating ischemia in the CNS and cardiovascular system. To investigate the consequences of reperfusion on the ENS, two models of ischemia-reperfusion injury in vitro were used: glucose deprivation followed by re-supply; and hypoxia followed by normoxia.

3.3.1 Resupply of glucose following deprivation causes acute neuron death in vitro

As previously described, complete deprivation of glucose for 24 hr causes non-selective damage (Fig. 4C). However, after only 16 hr of glucose deprivation, no loss of cells or axonal damage was observed (Fig. 8B,D). Following an initial 16 hr period of glucose deprivation, glucose was resupplied to co-cultures at the same concentration of glucose that was available in control conditions. After 1 hr resupply of glucose, neurons were lost and the axons appeared broken and much shorter (Fig. 8C). Neuron survival decreased to 64.5 ± 2.6% of control (n=23, p<0.001, Fig. 8D). Moreover, neuron loss did not progress further in greater lengths of time after resupply of glucose, indicating that acute neuron loss occurred soon after resupply of glucose. Neuron loss was not specific to a neuron type, since nitrergic neurons composed 24.0 ± 3.0% (n=3, p>0.05) of the neuron population, similar to control.

Total cell number was not significantly affected by glucose deprivation and resupply (Fig. 8D). However, analysis of glial cells revealed a negative trend in glial number after glucose deprivation and resupply. After resupply of glucose, glial cells formed 25.0 ± 3.9% (n=3, p>0.05) of total cell number, which is non-significantly lower than control.

In inflammation, ischemia may occur for varying amounts of time before subsequent reperfusion. In other situations, such as in surgery or transplantation, the period of ischemia is
Fig. 8. Resupply of glucose following a brief period of deprivation caused acute selective neuron loss in vitro, while smooth muscle cells were unaffected. A-C. Representative composite images of neuron bodies expressing HuD (green), pan-neuronal marker SNAP-25 (red) and all nuclei stained with Hoechst (blue) in a control co-cultures (A), after 16 hr of complete glucose deprivation (B) and after 16 hr of glucose deprivation followed by a 1 hr resupply of glucose (C). Glucose deprivation for 16 hr did not cause any neuron loss or visible damage to the axonal network. After 1 hr resupply of glucose, axons were shortened and broken and neuron number was decreased. Scale bars, 25 µm. D. Neuron and total cell number relative to control in vitro after 16 hr glucose deprivation and resupply, demonstrating acute neuron loss upon resupply, while accessory cells were unaffected. (n=23; *P<0.001 vs control; two-way repeated measures ANOVA with Bonferroni post-test).
monitored and minimized. Therefore, whether shorter periods of glucose deprivation would still invoke damage after resupply was tested. After only 2 hr of glucose deprivation, followed by 1 hr of resupply of glucose, neuron survival was reduced to 72.8 ± 1.5% of control (n=3, p<0.05). Therefore, glucose deprivation followed by a short resupply of glucose caused acute selective neuron loss.

3.3.2 Return to normoxia following hypoxia does not cause neuron death in vitro

As previously discussed, tissue experiencing ischemia may also face a hypoxic challenge. Subsequent reperfusion can potentially return the tissue to normoxia. These conditions were modelled in vitro by exposing co-cultures to 1% O₂ in a hypoxic chamber and then returning the culture to normoxia. Similar to the previous glucose deprivation and resupply model, a time point was chosen at which no damage is yet seen to neurons or surrounding cells. After 1.5 hr of hypoxia followed by 1 hr of normoxia, no visible damage was apparent and neuron survival was comparable to control (96.2 ± 21.9%, n=3, ns). This model was repeated with 4 hr, 8 hr, and 24 hr exposure to hypoxia prior to return to normoxia for 1 hr. In each case, return to normoxia did not cause any greater neuronal damage than was already present due to the initial hypoxic challenge. This indicates that neuronal damage in ischemia-reperfusion injury is not due to resupply of oxygen, but instead by glucose.

3.3.3 Resupply of glucose following deprivation causes acute neuron loss ex vivo

Both models of ischemia-reperfusion injury were also tested in ex vivo whole mounts of the SM/MP layers of young adult rat. In control whole mounts, neuron density was 142.1 ± 11.2 neurons/mm (n=4). After 16 hr of glucose deprivation, whole mounts appeared similar to control
(Fig. 9A,C), with neurons appearing in unbroken chain-like structures, 2-3 neurons wide, surrounded by glial extensions in a network. Similar to control, neuron density after 16 hr of glucose deprivation was 142.7 ± 12.7 neurons/mm (n=3). Following 16 hr of initial glucose deprivation, resupply of glucose for 1 hr caused a loss of neurons, while surrounding glial cells remained intact, creating gaps of HuD-immunonegative areas in the chain-like structure (Fig. 9B,D). After 1 hr resupply of glucose subsequent to deprivation, neuron density was significantly reduced to 97.0 ± 6.5 neurons/mm (n=4, p<0.05, Fig 9E). Similar to results in vitro, though, exposure to hypoxia followed by return to normoxia did not cause acute neuronal damage. This further confirmed that neurons are vulnerable to the effects of ischemia and reperfusion, and that glucose is responsible for that damage, rather than oxygen.

3.4 GDNF neuroprotection

Neurotrophins have been shown to improve the outcomes of otherwise neurotoxic challenges (Shang et al., 2010; Duarte et al., 2012). Therefore, GDNF, an important neurotrophin of the ENS, was tested for similar effects in metabolic challenge.

3.4.1 Application of exogenous GDNF is neuroprotective in metabolic inhibition

To test the hypothesis that GDNF may be neuroprotective in conditions of metabolic inhibition, exogenous GDNF was applied to primary co-cultures at a concentration of 50 ng/mL. For this, co-cultures exposed to metabolic inhibition by DNP or reduced glucose were compared to co-cultures treated with GDNF (50 ng/mL) applied 8 hr prior to or simultaneously with the metabolic challenge, as described in the Methods section. Application of 0.75 mM DNP to co-cultures for 24 hr showed a significant reduction in neuron survival to 62.9 ± 14.4% (n=3,
Fig. 9. Resupply of glucose following deprivation caused acute neuron loss in \textit{ex vivo} whole mounts. A-B. Representative images of neuron bodies expressing HuD in whole mounts of SM/MP layers of juvenile rat colon in control (A) and after 16 hr glucose deprivation followed by 1 hr resupply of glucose (B). C-D. Representative composite images of neuron bodies expressing HuD (green) and glial cells expressing GFAP (red) in SM/MP whole mounts in control (C) and after 16 hr glucose deprivation followed by 1 hr resupply of glucose (D). In control conditions, neurons were abundant and appeared in unbroken chains, enveloped by glial cells. In glucose deprivation and resupply, neurons were lost, while glial cells were unaffected, creating visible holes in the chain appearance (arrow). Scale bars, 25 µm. E. Neuronal density (neurons/mm) in whole mounts after 16 hr in specific conditions (n=3; *P<0.001 vs. control; one-way ANOVA with Bonferroni post-test).
Fig. 10. GDNF was neuroprotective against metabolic inhibition in vitro. Co-cultures were exposed to metabolic challenge with or without treatment with exogenous GDNF or prior transfection with a GDNF-expressing plasmid. A-B,D-E. Representative composite images of neuron bodies expressing HuD (green), pan-neuronal marker SNAP-25 (red) and all nuclei stained with Hoechst (blue) in co-cultures after 24 hr treatment with 1 mM DNP (A), with application of 50 ng/mL exogenous GDNF both 8 hr prior to and simultaneous to treatment with 0.75 mM DNP (B), 80% reduction of glucose concentration to 5 mM (D), and 80% reduction of glucose concentration in a culture transfected with a plasmid producing GFP-GDNF (E). In treatment with DNP or reduced glucose, neurons were selectively lost and axons appeared visibly damaged. GDNF, whether applied exogenously (B) or produced endogenously by transfection (E) prevented neuron loss in metabolic challenge and supported extensive axon networks. Scale bars, 25 µm. C. Neuron number relative to control in vitro after 24 treatment with specific metabolic challenge with or without application of exogenous GDNF, demonstrating neuroprotective effects of GDNF. (n=3-6; *P<0.05 vs control; one-way repeated measures ANOVA with Bonferroni post-test). F. Neuron number relative to control in vitro after 24 hr treatment with specific metabolic challenge with or without prior transfection with a plasmid producing GDNF, demonstrating neuroprotective effects of GDNF. (n=3; *P<0.05; two-tailed paired Student’s t-test).
of control and surviving neurons have shortened and broken axons (Fig. 10A). This was not substantially altered by co-application of GDNF. However, both pre- and co-treatment of GDNF combined with exposure to DNP completely prevented neuron loss and maintained the extensive axonal network observed in healthy cultures (Fig. 10B). Pre- and co-treatment of GDNF in addition to DNP treatment increased neuron survival to 90.3 ± 8.9% (n=3, p<0.05) of control (Fig. 10C). GDNF had similar neuroprotective effects in reduced glucose conditions, where pre-treatment with GDNF completely prevented the damage done by glucose reduction, a trend that neared significance (n=6, p=0.15; Fig. 10C). This demonstrates that prior addition of GDNF to co-cultures is able to prevent neuron loss due to metabolic challenge.

3.4.2 Increased GDNF expression by transfection prevents damage by metabolic inhibition

We have previously shown that ISMCs are the source of GDNF in neonatal and adult rats in vitro and in vivo (Gougeon et al., 2013). To further study the effect of GDNF, co-cultures were transfected with a plasmid expressing GFP-GDNF and followed this with metabolic challenge by DNP or replacement of glucose with OMG. Prior to metabolic challenge, GFP expression was visually assessed to ensure effective transfection. 16 hr after transfection, a small number of cells were fluorescent, indicating GFP expression. Co-cultures transfected with plasmid expressing GFP alone showed no neuron loss relative to control, meaning that the transfection process alone does not significantly affect outcome.

Transfection of co-cultures with GFP-GDNF was then used to demonstrate that this method of delivery also conferred significant neuroprotection against metabolic challenge. Reduction of glucose caused significant neuron loss and axonal damage (Fig. 10D) and 24 hr treatment with 80% reduction in glucose reduced neuron survival in non-transfected cultures was
reduced to 58.1 ± 4.4% (n=3, p<0.001) of control. However, in transfected co-cultures, neuron survival in reduced glucose conditions was not decreased and remained at control levels (104.1 ± 2.2%, n=3). An extensive axonal network was present, showing lack of damage (Fig. 10E,F). Similar results were observed in treatment with DNP (Fig. 10F). Therefore, similar to exogenous GDNF, increasing endogenous levels of GDNF through transfection can also prevent the neurotoxic effects of metabolic inhibition.

3.4.3 Application of exogenous GDNF is neuroprotective against ischemia-reperfusion injury

The ability of GDNF to protect enteric neurons during challenge was also tested in an in vitro model of ischemia-reperfusion injury. As described earlier, neuronal damage was not observed after the initial insult of 16 hr of glucose deprivation, but a 1 hr period of resupply following deprivation caused neuron loss and axonal damage (Fig. 11A), and neuron survival decreased to 65.1 ± 2.1% of control (n=4, p<0.001, Fig. 11D). The effect of exogenous GDNF, 50 ng/mL was tested with application either 8 hr prior to and/or simultaneously with the resupply of glucose. Co-treatment of exogenous GDNF with resupply of glucose was neuroprotective, significantly increasing neuronal survival to 80.1 ± 7.1% of control (n=4, p<0.05). Pre-treatment with exogenous GDNF caused further prevention of neuron loss and the greatest effect was observed when co-cultures were both pre- and co-treated with GDNF, completely preventing neuron loss (92.7 ± 18.8%, n=4, p<0.05) upon resupply of glucose following deprivation, and maintaining a healthy axonal network (Fig. 11B,D).
Fig. 11. GDNF was neuroprotective in glucose deprivation and resupply \textit{in vitro}. Co-cultures were deprived of glucose for 16 hr, followed by a 1 hr resupply of glucose, with or without application of exogenous GDNF or prior transfection with a GDNF-expressing plasmid. A-C. Representative composite images of neuron bodies expressing HuD (green), pan-neuronal marker SNAP-25 (red) and all nuclei stained with Hoechst (blue) in co-cultures after 16 hr glucose deprivation, followed by 1 hr resupply of glucose (A), with application of 50 ng/mL exogenous GDNF 8 hr prior to and simultaneous with resupply of glucose (B), or with prior transfection of cultures with plasmid expressing GDNF (C). In the ischemia-reperfusion model, neurons were lost and the axons were visibly damaged. Both exogenous and endogenous GDNF prevented neuron loss and supported an extensive axon network. Scale bars, 25 µm. D. Neuron number relative to control \textit{in vitro} after 16 hr glucose deprivation followed by resupply of glucose, with or without application of exogenous GDNF, demonstrating the neuroprotective effects of GDNF. (n=4; *P<0.05 vs. control; Friedman test with Dunn’s multiple post-test comparison). E. Neuron number relative to control after 16 hr glucose deprivation followed by resupply of glucose. Prior transfection with a plasmid expressing GDNF resulted in increased survival relative to mock transfected controls (n=; *P<0.001; two-tailed paired Student’s t-test).
3.4.4 Increased GDNF expression by transfection prevents damage by ischemia-reperfusion

Similar results were obtained when GDNF availability was increased endogenously by transfection, rather than exogenous application. In GFP-GDNF transfected co-cultures that were first glucose deprived for 16 hr and then had glucose resupplied for 1 hr, neuron loss was completely prevented (96.7 ± 7.8%, n=3) and the axonal network between neurons and surrounding cells showed no evidence of damage (Fig. 11C,E). Hence, GDNF can prevent the neurotoxic outcomes of an ischemia and reperfusion protocol in vitro.

3.5 Preconditioning via HIF1-α has differential effects in ischemia and ischemia-reperfusion models in vitro

A transient sub-threshold exposure to hypoxia prior to subsequent challenge (preconditioning) has been shown to be protective against neuronal damage in the CNS in ischemia. In order to determine if preconditioning could be protective for the ENS as well, co-cultures were exposed to 5% O₂ for 4 hr, which had been previously determined to not cause neuron loss. Then, 16 hr later, a metabolic challenge of DNP, acute hypoxia, or glucose deprivation was applied.

3.5.1 HIF1-α upregulation is neuroprotective in in vitro ischemic models

As shown above, after 24 hr in 80% reduced glucose conditions, neuron survival in co-cultures was reduced by about 40%. Not only were neurons lost, but axons were shortened and damaged (Fig. 12A). However, preconditioning was highly effective in preventing damage in reduced glucose challenge, and a healthy axonal network was restored (Fig. 12B). As well, neuron survival returned to control level (95.6 ± 7.2%, n=5, p<0.05, Fig. 12D). Overall,
Fig. 12. Hypoxic preconditioning was neuroprotective via HIF1-α induction in metabolic challenge in vitro. Co-cultures were exposed to sub-threshold hypoxia prior to metabolic challenge, with or without inhibition of HIF1-α by chetomin. A-C. Representative composite images of neuron bodies expressing HuD (green), pan-neuronal marker SNAP-25 (red) and all nuclei stained with Hoechst (blue) in co-cultures after 24 hr treatment with 80% reduction in glucose concentration to 5 mM (A) with both reduction in glucose and 4 hr exposure to 5% O₂ 16 hr prior to treatment (B) and with reduction in glucose, prior hypoxic preconditioning and treatment with 10 nM chetomin (C). Scale bars, 25 µm. D. Neuron number relative to control in vitro in specific combinations of metabolic challenge, hypoxic preconditioning and treatment with 10 nM chetomin. Hypoxic preconditioning prevented neuron loss in metabolic challenge and inhibition of HIF1-α by chetomin ablated the protective effects of preconditioning (n=4; *P<0.05; one-way repeated measures ANOVA with Dunnett’s multiple comparison test).
preconditioning prevented the neurotoxic effects of metabolic inhibition by either glucose deprivation, hypoxia or DNP (Fig. 12D).

Elsewhere, HIF1-α activity has been shown to be responsible for many of the neuroprotective effects of preconditioning (Bergeron et al., 2000). To test whether HIF1-α was involved here, co-cultures were preconditioned and then received chetomin, an inhibitor of HIF1-α (Marsboom et al., 2012), simultaneously with the metabolic challenge. If HIF1-α upregulation was responsible for the neuroprotective effects of preconditioning in metabolic challenge, the neurotoxic effects of metabolic challenge should reemerge with chetomin treatment. Indeed, in preconditioned cultures that received treatment with 10 nM chetomin and a reduced glucose challenge, significant neuron loss occurred (69.1 ± 4.1% of control, n=5, p<0.05, Fig. 12D, and there was substantial damage to the axonal network (Fig. 12C). Similar results were found after challenge with DNP and hypoxia. Thus, inhibition of HIF1-α completely nullified the neuroprotective effects of preconditioning in metabolic inhibition.

3.5.2 HIF1-α upregulation is maladaptive in an in vitro ischemia-reperfusion model

HIF1-α has been mostly observed to have beneficial effects in metabolic challenge, as part of the body’s adaptive response to ischemia. Yet, a few studies have investigated whether HIF1-α then becomes maladaptive in ensuing reperfusion (Kannan et al., 2010). Therefore, co-cultures were preconditioned as before in conjunction with the glucose deprivation and resupply in the in vitro model of ischemia-reperfusion. As discussed previously, 16 hr of glucose deprivation does not show any visible damage, but a 1 hr subsequent resupply of glucose causes axonal damage and about a 35% neuron loss (Fig. 13A,D). Interestingly, though preconditioning was neuroprotective in the in vitro ischemic models, preconditioning was not able to prevent
neuronal damage in glucose resupply. Axons remained damaged and neuron survival was 64.1 ± 11.9% (n=3, n.s.) of control, similar to non-preconditioned cultures after glucose deprivation and resupply (Fig. 13B,D).

To examine the role HIF1-α may play in the damage observed in glucose resupply, and whether it is the reason why preconditioning is not helpful in glucose resupply, co-cultures were treated with chetomin as well. First of all, preconditioned cultures were treated with 10 nM chetomin at the onset of the initial 16 hr glucose deprivation. In these cultures, neuron survival did not improve (Fig. 13D). Secondly, preconditioned cultures were treated with chetomin after the initial 16 hr glucose deprivation, simultaneously with the 1 hr resupply of glucose. In these cultures, neuron survival significantly improved and the axonal network was partially restored (Fig. 13 C). Neuron survival returned to 93.5 ± 17.1% (n=3, p<0.05) of control. Thus, preconditioning is helpful in metabolic inhibition by upregulating HIF1-α activity, which then becomes maladaptive upon restoration of glucose.
Fig. 13. Neuron loss in glucose deprivation and resupply is prevented by hypoxic preconditioning combined with inhibition of HIF1-α during the resupply of glucose in vitro.

A-B. Representative composite images of neuron bodies expressing HuD (green), pan-neuronal marker SNAP-25 (red) and all nuclei stained with Hoechst (blue) in co-cultures after 16 hr glucose deprivation, followed by 1 hr resupply of glucose (A), alongside both 4 hr exposure to 5% O₂ 16 hr prior to glucose deprivation and treatment with 10 nM chetomin upon reperfusion (B). Scale bars, 25 µm. C. Neuron number relative to control in vitro after 16 hr glucose deprivation and 1 hr resupply of glucose, and with varying combinations of hypoxic preconditioning and treatment with 10 nM chetomin. Hypoxic preconditioning alone did not prevent neuron loss in glucose deprivation and resupply, but hypoxic preconditioning followed by inhibition of HIF1-α by chetomin upon resupply of glucose was neuroprotective (n=5; *P<0.05; one-way repeated measures ANOVA with Dunnett’s multiple comparison test).
Chapter 4

Discussion

Integrity of the ENS is vitally important to life. By coordinating motility, the ENS allows for the proper digestion and absorption of nutrients and expulsion of waste. Yet, intestinal inflammation results in significant myenteric neuron loss, threatening the ability to perform these functions (Poli et al., 2001). A greater understanding of the mechanisms behind neuron loss in inflammation will lead to methods of protection, potentially preventing post-inflammatory motility disorders. Therefore, using in vitro models, this study examined the possibility of metabolic inhibition due to ischemia as a major factor behind ENS damage in IBD. Furthermore, the impact of ensuing reperfusion, as might occur during remitting and recurring IBD or during surgery, on enteric neurons was investigated in vitro. Methods of neuroprotection in these conditions were also explored, including use of the neurotrophin GDNF and hypoxic preconditioning.

4.1 Significance of increased HIF1-α activity in vivo in TNBS-colitis

The possibility of ischemia occurring in inflammation was assessed by HIF1-α expression. HIF1-α is an important part of the cellular response to hypoxia, and is therefore recognized as a marker for hypoxia (Sharp & Bernaudin, 2000). Furthermore, in previous studies, ischemia has been correlated with an increase in HIF1-α presence and activity (Jin et al., 2000; Dharap et al., 2009). Thus, in this study, HIF1-α expression was examined in vivo in TNBS-induced colitis. As expected, cells in the mucosa were HIF1-α positive, even in control conditions. However, HIF1-α expression was also found to be upregulated in inflammation in the
SM/MP layers in both SMCs and myenteric neurons. Importantly, HIF1-α was translocated from the cytoplasm into the nucleus in TNBS-induced colitis, indicating an increase in HIF1-α’s transcriptional activity. The most direct conclusion of these findings is the SM/MP layers are experiencing hypoxia during inflammation. This would corroborate previous literature positing that ischemia occurs in intestinal inflammation (Funayama et al., 1999; Collins CE et al., 1997). However, it was not yet understood whether ischemia in inflammation would reach and affect the deeper SM/MP layers, which is a novel finding of this study.

Nevertheless, alternative causes aside from hypoxia for upregulation of HIF1-α must be considered. One possibility is that the inflammatory process directly causes upregulation of HIF1-α, without the presence of ischemia or hypoxia (Westra et al., 2007; Haddad & Land, 2001). Inflammatory cytokines, such as TNF-α and IL-1, have been found to be upregulated in the SM/MP layers during inflammation (Collins SM et al., 1997, Shi & Sarna, 2005), which could then cause activation of HIF1-α. One study in 2003 found that application of IL-1β to cultures of the A549 cell line, adenocarcinomic human alveolar basal epithelial cells, caused an increase in HIF1-α (Jung et al.). Conversely, an earlier study in 2001 found that HIF1-α protein content was greater in in vitro inflammatory cells treated with TNF-α, but not IL-1 (Albina et al.). In vivo, Albina et al. found that HIF1-α was greater in cells around wounds, which they confirmed was due to hypoxia using Hypoxyprobe (2001). Thus, upregulation of HIF1-α by inflammatory cytokines is possible. However, several notes about these studies must be made. First of all, both of these studies observed cytokine induction of HIF1-α in in vitro cultures. Secondly, the studies disagreed on whether IL-1 is capable of inducing HIF1-α, demonstrating that IL-1 is not as reliable an inducer of HIF1-α as compared to hypoxia. Thirdly, neither of the studies examined where the HIF1-α was located, simply that protein and mRNA content was
increased. It is possible that inflammatory cytokines may increase HIF1-α production, without increasing transcriptional activity by translocation to the nucleus. Lastly, even though inflammatory cytokines were concluded to cause induction of HIF1-α by these studies, the possibility of hypoxia also being present was not assessed.

Therefore, considering the observation of increased HIF1-α presence in the nuclei in inflammation was made in vivo in this study, the most likely cause remains hypoxia due to ischemia. Nonetheless, taking into account the possibility of direct induction by inflammatory cytokines, a conservative conclusion would still be that the cells are responding in a similar fashion as to hypoxia and are experiencing metabolic challenge. In the future, the relationship between inflammatory cytokines and HIF1-α should be investigated by observing whether inhibiting cytokine activity, such as with anti-TNF-α antibody, infliximab, has an effect on HIF1-α activity.

4.2 Comparison of in vitro models of ischemia and ischemia-reperfusion

One of the goals of this study was to create in vitro models of ischemia in order to examine the effect of ischemic challenge on enteric neurons. The three models chosen were glucose deprivation, hypoxia and DNP. All three of these models have been used substantially in previous research in other body systems, varying concentration and exposure time (Murata et al., 2001; Banasiak & Haddad, 1998; Le et al., 2002). By utilizing in vitro models, different facets of ischemia can be isolated. However, the differences and similarities between the in vitro model and the expected conditions in vivo are important to note in order to accurately interpret results.

Both glucose deprivation and hypoxia are expected outcomes of ischemia. However, the amount of sugar or oxygen that an enteric neuron might have access to during ischemia in vivo is
not known and is expectedly quite variable. At first, we demonstrate that neuron loss is progressive as sugar availability decreases. For further experiments, 5 mM glucose for 24 hr was chosen since it was the most extreme demonstration of the impact of glucose deprivation on neurons, while not yet affecting surrounding cells. In vivo, depending on the severity and length of ischemia, it is reasonable to posit that the smooth muscle layers may become affected. An important difference between hypoxia and glucose deprivation to note is that glucose deprivation prevents the cell from creating energy even through glycolysis. Hypoxia limits oxidative phosphorylation, but the cell still has access to sugar for glycolysis. On the other hand, DNP uncouples the electron transport chain, therefore also limiting oxidative phosphorylation. While DNP itself will not be present in ischemia, mitochondrial dysregulation and uncoupling is an established outcome of ischemia, as will be discussed in the next section. Overall, the models used do give important insights into the possible consequences of ischemia, while also demonstrating the impact of isolated forms of metabolic inhibition.

4.3 Role of mitochondria in neuronal vulnerability

This study repeatedly found that enteric neurons were more vulnerable than surrounding SMCs and glial cells to metabolic challenge, both in models of ischemia and ischemia-reperfusion. In comparison to previous literature, our co-culture model provides a unique comparison of neurons and SMCs, which studies of the CNS often lack. The impact of ischemia, and particularly reperfusion, on the SM/MP layers has not been extensively studied. However, in the few studies of ischemia and ischemia-reperfusion injury in the SM/MP layers of the intestine in animal models, most have found damage to SMCs, enteric neurons and interstitial cells of Cajal (ICC), including loss of myenteric neurons, thickening of the muscle layers and activation
of apoptotic pathways in SMCs and ICC (Mei et al., 2009; Lindestrom & Ekblad, 2004). Yet, an issue in these studies may be the choice of time-points for observation – both performed SMAO for 60 min followed by reperfusion for 12 hr to 10 days. In our hypoxia model, 24 hr of exposure led to a decrease in both neurons and total cell number. However, at only 4 hr of exposure, only neurons had been lost. This is an important observation because in occurrences of ischemia-reperfusion in surgery, the length of time may be short enough to not affect the muscle layers and there may be no visible damage, while the vulnerable ENS is actually affected.

Several factors behind this observed neuronal vulnerability may be possible, all relating to metabolic demand and mitochondrial susceptibility to dysregulation. Compared to other cell types, neurons require more energy to maintain its resting potential along axons and generate action potentials (Waldvogel et al., 2000). It was found that the difference between the energy required in order to maintain activity and the maximum amount of energy the cell was capable of producing was smaller in CNS neurons than in SMCs (Ames, 2000). Thus, lower levels of metabolic inhibition have a greater impact on neurons.

Additionally, neurons are also particularly sensitive to mitochondrial dysfunction. In previous studies, low levels of uncouplers, such as DNP used in this study, led to the production of superoxide free radicals (Nicholls, 2008, Murakami et al., 1998). Furthermore, many studies have implicated mitochondrial membrane potential, mainly associated with calcium regulation, to have an important role in death in both ischemia and reperfusion. It has been established that intracellular calcium rises steadily and slowly during ischemia until a critical concentration was reached, an effect that was mitigated by preconditioning (Ylitalo et al., 2000, Szydlowska & Tymianski, 2010). Other researchers have examined the process in more detail, concluding that as cytosolic calcium rises initially in neurons, the mitochondria sequester calcium in an attempt
to cope, but this then leads to reactive oxygen species (ROS) generation (Starkov et al., 2004). In fact, one study concludes that the key event in neuronal ischemic death is the “uncoupling of the mitochondrial respiratory chain, which may be associated with mitochondrial permeability transition” (Frantseva et al., 2001). Though intracellular calcium concentration rises slowly during ischemia, an acute excitotoxic influx of calcium occurs during reperfusion (Ylitalo et al., 2000). This effect was found to be preventable by the use of calcium channel blockers in in vitro experiments (Li et al., 2007).

However, these studies used CNS neurons and many noted variability even between different subsets of CNS neurons (Nicholls & Budd, 2000). Furthermore, many studies also implicated glutamate excitotoxicity as a variably important step in the process (Nicholls, 2008; Murakami et al., 1998). However, glutamate is not known to be an important excitatory neurotransmitter in the ENS as in the CNS. Thus, our findings provide novel insight into the increased metabolic demand and mitochondrial vulnerability of enteric neurons similar to neurons of the CNS.

4.4 Glucose, not oxygen, is responsible for ischemia-reperfusion injury

The cause of ischemia-reperfusion injury has been an extensive area of research for the past two decades. It has been established that ischemia-reperfusion injury is mostly attributed to oxidative stress from overproduction of oxygen free radicals (Collard & Gelman, 2001; Kaminski et al., 2002). Reperfusion also attracts inflammatory cells, including neutrophils, while ROS causes direct damage and induces release of pro-inflammatory cytokines (Collard & Gelman, 2001). In both in vitro and in vivo experiment, antioxidants have been found to be an important therapy in ischemia-reperfusion, including the use of hydrogen gas that will bind to
hydroxyl radicals (Fukuda et al., 2007). Hypoxia-reoxygenation is a common in vitro model of ischemia-reperfusion, as was done in this study. Isolated from other consequences of ischemia-reperfusion such as the presence of inflammatory cells, hypoxia-reoxygenation has been repeatedly found to be sufficient to cause an increased amount of ROS and cellular injury (Karmazyn et al., 1993; Li & Jackson, 2002). In mixed cultures of cortical neurons and glia, cortical neurons were found to be significantly more vulnerable than glia to hypoxia-reoxygenation (Wang et al., 2002). This may partly be due to free radical formation causing activation of the NO synthase, leading to added damage from excitotoxicity (Cazavieille et al., 1993). In the end, reoxygenation subsequent to hypoxia or anoxia is considered to be the main culprit in the generation of ROS in ischemia-reperfusion.

In this study, however, two in vitro models of ischemia-reperfusion were used: depriving and resupplying glucose and hypoxia-reoxygenation. This allows for two major components of ischemia and reperfusion to be isolated from one another. Interestingly, glucose deprivation and resupply caused acute selective neuronal damage, while oxygen deprivation and resupply did not. Based on the previous literature discussed, this is unexpected.

Nevertheless, there are studies that would substantiate and explain our findings. First of all, glucose can be responsible for the oxidative stress seen in ischemia-reperfusion, similar to what is observed in hypoxia-reoxygenation. In humans, greater preprandial to postprandial changes in blood glucose led to increase oxidative stress, as estimated by urinary excretion rates of 8-iso prostaglandin F$_{2\alpha}$ (Monnier et al., 2006). In cultures of human endothelial cells, large acute fluctuations in glucose concentration caused an increase in superoxide production and apoptosis (Piconi et al., 2006). Thus, an acute fluctuation in glucose concentration is sufficient to cause ROS generation. Secondly, hypoxia-reoxygenation directly induces production of
antioxidants. Microarray analysis of renal epithelial cells in culture exposed to hypoxia-reoxygenation showed an increase in antioxidant transcription factor Nrf2, which led to attenuation of ROS production (Leonard et al., 2006). This is proposed as one mechanism by which hypoxic preconditioning is beneficial. Thirdly, ischemia-reperfusion injury has been observed to be worsened by previous repeated fluctuations in glucose concentration. The cardiac damage due to ischemia-reperfusion was found to be greater in mice that received single daily gavages of sucrose compared to placebo, an effect that was diminished by co-gavage with acarbose (Frantz et al., 2005). In humans, ischemia-reperfusion injury tends to be worse in uncontrolled diabetes (Ceriello et al., 2008; Chang et al., 2012). Therefore, acute glucose fluctuations are capable of causing damage through ROS production, while not causing any parallel increase in antioxidants. In our cultures, hypoxia-reoxygenation may not have caused neuronal loss because of parallel increases in both ROS and antioxidants.

4.5 Mechanisms of neuronal death in ischemia and ischemia-reperfusion injury

In the in vitro ischemic models, neurons died gradually and consistently over time. However, in the in vitro ischemia-reperfusion model, neuron death occurred acutely in a short span of time. Neurons that did not die within the first hour had not died by further time-points. These observations raise important questions about how enteric neurons die in various conditions.

Both ischemia and ischemia-reperfusion injury is often associated with apoptotic pathways in other parts of the body, including the CNS, liver and the heart (Jaeschke, 2003). However, the molecular mechanisms of death in neurons, particularly enteric neurons, are not as well understood. In cultures of SHY5Y neuroblastoma cells, glucose deprivation caused death by
apoptosis, as evidenced by expression of apoptotic genes, including caspases (Russo et al., 2012). At the same time, caspase-independent cell death has also been observed, where caspase inhibitors did not entirely prevent neuron loss in the hippocampus in a bilateral common carotid artery occlusion model (Zhan et al., 2001). In an ischemia-reperfusion model applied to the rat retina, neuronal death was determined to be caspase-independent and inhibited by necrostatin-1, which lead to the conclusion that necroptosis was occurring (Rosenbaum et al., 2010). Thus, the mechanism of death in ischemia and ischemia-reperfusion in the CNS and PNS are still being examined and appears to be variable based on a number of factors.

Investigation into the mechanism of death in the ENS yields similar conflicting results. First of all, in in vivo models of ischemia-reperfusion, the time at which neuron damage and death is observed varies widely, ranging from 1 hr, similar to our findings (Linestrom & Eckblad, 2004) to 24 hr (Rivera et al., 2009). Secondly, some studies have also noted variation in the responses of different neuron types, such as selective damage to nitricergic neurons, which was not observed in our study (Rivera et al., 2009). As for mechanism of death, some studies have posited that neurons, SMCs and ICC all die by apoptosis in reperfusion injury, as evidenced by TUNEL staining (Mei et al., 2009, Rivera et al., 2011). However, this does not answer whether it is caspase independent or dependent. Examining our own cultures, the health of surrounding accessory cells in the co-cultures after neuron loss had already occurred indicates that necrosis is not likely. This does not exclude the possibility of necroptosis or autophagy.

4.5 Mechanisms of GDNF neuroprotection in ischemia and ischemia-reperfusion

As previously discussed, GDNF has been consistently shown to protect against injury in ischemic conditions in the CNS. Though GDNF’s actions may differ in the CNS and ENS, this
study demonstrates that GDNF can also protect against neuronal damage of the ENS *in vitro* in models of ischemia. Moreover, our study demonstrated GDNF neuroprotection in an *in vitro* model of ischemia-reperfusion. Considering the mechanism of death in prolonged ischemia is likely to differ from that in acute ischemia-reperfusion, the mechanism behind GDNF neuroprotection requires further examination. It may be that GDNF promotes neuronal survival in a singular way that applies in both conditions, or is involved in multiple neuroprotective pathways.

GDNF is known to combine with GDNF family receptor alpha 1 (GFRα1) and then bind with the Ret receptor tyrosine kinase to enact a cascade (Durbec *et al.*, 1996). Downstream actors in this pathway continue to be identified. One key player that requires further examination is Akt, since it is an important signaling molecule for cell survival. Previously, Akt phosphorylation was observed to be reduced in an *in vivo* and an *in vitro* rat model of diabetes, in which there was also myenteric neuron loss. In the *in vitro* model, application of exogenous GDNF was able to prevent myenteric neuron loss by increasing Akt phosphorylation (Anitha *et al.*, 2006). However, the study did not block Akt phosphorylation to assess whether GDNF remains effective.

As well, whether other neurotrophins are capable of being similarly neuroprotective in metabolic inhibition should be investigated. In some studies, other neurotrophins, such as nerve growth factor (NGF), neurotrophin-3 (NT-3), or brain-derived neurotrophic factor (BDNF), were not able to support neuron survival in postnatal enteric neurons similar to GDNF (Rodrigues *et al.*, 2011). In other studies, these same neurotrophins were protective in inflammation and promoted postnatal ENS plasticity (Reinshagen *et al.*, 2000; von Boyen *et al.*, 2002). The effects
of other GDNF family members, such as neurturin, will also reveal further information on the signaling molecules within the GDNF pathway responsible for neuroprotection.

Lastly, though not examined in this study, previous research indicates that GDNF applied after damage may also be beneficial by supporting axon outgrowth that would compensate for lost innervation (Rodrigues et al., 2011). As previously mentioned, inflammatory cytokines are known to induce GDNF expression and secretion (Gougeon et al., 2013; von Boyen et al., 2006). Thus, in vivo, GDNF may be responsible for the cessation in neuron loss after day 2 TNBS and be an important mediator of the axonal outgrowth seen as a later event in the TNBS model.

4.6 Differential role of preconditioning and HIF1-α in ischemia and ischemia-reperfusion

In our in vitro models of ischemia, hypoxic preconditioning was beneficial to myenteric neuron survival. The beneficial effects of hypoxic preconditioning in ischemia, as previously discussed, has been well established over the past ten years in many systems including the heart, liver, kidney and CNS (Lasley et al., 1993; Hu et al., 2008). Several mechanisms have been found to be involved in preconditioning. First of all, brief hypoxia is associated with upregulation of antioxidant enzymes (Leonard et al., 2006). In an in vitro model, using oxygen-glucose deprivation, cultures that had received preconditioning had a 54% increase in glutathione peroxidase, 73% increase in glutathione reductase, and a 32% increase in Mn superoxide dismutase (Arthur et al., 2004). Secondly, preconditioning induces HIF1-α activity, which then allows for important genes to be transcribed that will aid in coping with the metabolic challenge. For example, nNOS was actually recognized as having an important role in preconditioning’s benefits before it was realized to be a transcriptional target of HIF1-α (Gidday et al., 1999). In
mice, preconditioning with dimethylxalylglycine (DMOG), and inhibitor of HIF1-α breakdown, increases NOS production (Zhang et al., 2011). Furthermore, the benefits of preconditioning are partially mitigated by inhibiting NOS (Zhang et al., 2011; Mahfoudh-Boussaid et al., 2012). Hypoxic preconditioning also induces a low level of ROS, which was found to lower the threshold for HIF1-α translocation, allowing for a faster response in subsequent metabolic challenges (Liu et al., 2005). In agreement with previous research, the benefits of preconditioning were abolished when we inhibited the transcriptional activity of HIF1-α using chetomin, demonstrating that HIF1-α is an important mediator of preconditioning.

On the other hand, previous research about preconditioning and ischemia-reperfusion is less decisive than its consistent benefits in ischemia. Some studies demonstrate that preconditioning and particular HIF1-α is beneficial in ischemia reperfusion injury. In one study, hypoxic preconditioning 48 hr prior to a transient focal MCAO reduced infarct size by about 50% (Miller et al., 2001). In another study, inhibiting HIF1-α breakdown with DMOG significantly diminished the damage of ischemia reperfusion in the livers of mice (Schneider et al., 2010). One proposed mechanism by which hypoxic preconditioning can be beneficial for both ischemia and ischemia-reperfusion is through a negative feedback loop. Hypoxia was found to upregulate prolyl hydroxylase activity, which would increase HIF1-α breakdown (D’Angelo et al., 2003). Thus, after preconditioning, a future ischemic episode would cause a faster HIF1-α response, but in reperfusion increased prolyl hydroxylase is present to cope.

However, for every paper that demonstrates the beneficial effects of HIF1-α and preconditioning in reperfusion, there is one that demonstrates negative effects. Cursio and colleagues found that HIF1-α induction was associated with increased apoptosis in ischemia-reperfusion in rat liver (2008). Another study found that partial HIF1-α deficiency reduced
inflammatory cytokines in intestinal ischemia-reperfusion and improved outcome (Feinman et al., 2010). Interestingly, one study found that preconditioning in hyperoxia instead is beneficial in tMCAO in the rat brain, by inhibiting apoptosis (Li et al., 2009).

It has been suggested that hypoxic preconditioning is only beneficial during the ischemic portion of ischemia-reperfusion injury, but that in some cases this still leads to an overall improvement (Loor & Schumacker, 2008). This would substantiate the findings of this study. In the in vitro model of ischemia-reperfusion in this study, hypoxic preconditioning had no effect, perhaps because the beneficial effects during deprivation and negative effects during resupply nullify each other. Treatment with chetomin at the onset of glucose deprivation also had no effect. However, treatment with chetomin with the onset of glucose resupply partially mitigated the neuronal damage. By allowing the presence of HIF1-α during glucose deprivation and then inhibiting HIF1-α during the reperfusion alone, the neuron can cope with both stresses.

4.7 Conclusions

Using a combination of in vivo, ex vivo and in vitro approaches, this thesis examined the potential for ischemia and reperfusion to occur in the inflamed intestine, and the impact of that challenge on the ENS. Immunolabelling for HIF1-α in sections of rat intestine revealed increased expression and nuclear translocation in the inflamed intestine, indicating metabolic challenge perhaps due to ischemia. Reduced glucose, hypoxia and DNP all caused neuronal loss and axonal damage in in vitro co-cultures of SMCs, glia and enteric neurons and in ex vivo whole mounts of the SM/MP layers of rat intestine. Furthermore, resupply of glucose following glucose deprivation, as a model ischemia and reperfusion, caused acute neuronal loss, while hypoxia and reoxygenation did not cause a similar loss. Neuronal loss and axonal damage in metabolic
challenge was prevented by pre-treatment with GDNF. Lastly, hypoxic preconditioning was neuroprotective in models of ischemia *in vitro*, which was prevented by inhibition of HIF1-α. However, hypoxic preconditioning and HIF1-α becomes detrimental in a reperfusion model *in vitro*. These findings demonstrate the vulnerability of enteric neurons and the potential for damage in ischemia and reperfusion during inflammation or in other conditions, such as transplantations.
References


