Abstract

The gastrointestinal (GI) tract is subject to regulation by several neuronal networks, one of which is the sympathetic nervous system (SNS). Inflammatory bowel diseases (IBD), most importantly Crohn’s disease and ulcerative colitis, are chronic diseases of the GI tract that result in such functional symptoms as abdominal pain and diarrhea. These symptoms suggest an important role for dysregulation of the SNS in IBD, since this branch of the autonomic nervous system aids in regulation of blood flow, secretion and motility. Inflammatory cytokines that are elevated in the serum and tissue of IBD patients can have wide-ranging effects on neuronal function in vitro, and may be responsible for the functional alterations observed in vivo.

With these neuronal alterations in mind, we hypothesized that interleukin-17, a novel cytokine with a central role in the pathogenesis of IBD, modulates the properties of sympathetic neurons innervating the gastrointestinal tract. Using electrophysiological techniques and Ca\(^{2+}\) imaging, we examined the effect of IL-17 on currents passing through voltage-gated Ca\(^{2+}\) channels in neurons from the superior mesenteric ganglion, which innervates the gut, and found that IL-17 inhibited these currents. In parallel, we found that IL-17 enhances the growth of sympathetic neurites in vitro. These effects depend upon activation of the nuclear factor κB (NF-κB) pathway, and do not appear to require glial cells. Therefore, dysregulated neural function during IBD may be due to direct effects of IL-17 on sympathetic neurons.
Co-Authorship

The polymerase chain reaction and myeloperoxidase assay experiments were carried out by Ms. Shadia Neshat.
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Table of Contents

Abstract ............................................................................................................................................ ii
Co-Authorship .................................................................................................................................... iii
Acknowledgements ........................................................................................................................ iv
Table of Contents ............................................................................................................................... v
List of Figures ..................................................................................................................................... vii
Chapter 1 Introduction ....................................................................................................................... 1
  1.1 Sympathetic innervation of the gastrointestinal tract ............................................................... 1
  1.2 Neural regulation of GI function is altered during inflammation .......................................... 4
  1.3 Interleukin 17 ........................................................................................................................... 5
    1.3.1 Induction and maintenance of Th17 cells ...................................................................... 5
    1.3.2 Experimental evidence for the effects of IL-17 in the GI tract ................................... 6
    1.3.3 Clinical evidence for a role of IL-17 in IBD ................................................................. 7
    1.3.4 Potential significance .................................................................................................... 8
  1.4 Ca^2+ currents and synaptic transmission ............................................................................. 8
  1.5 Ca^2+ currents and neurite outgrowth .................................................................................. 10
  1.6 IL-17: A neurotrophic cytokine? ........................................................................................ 12
  1.7 Inflammation and increased nerve sprouting ................................................................... 14
  1.8 Unresolved issues ................................................................................................................. 16
  1.9 Hypothesis ........................................................................................................................... 16
Chapter 2 Methods ......................................................................................................................... 17
  2.1 Animal care ........................................................................................................................... 17
  2.2 IBD model ............................................................................................................................ 17
  2.3 Tissue collection .................................................................................................................. 17
  2.4 Reverse transcriptase PCR .................................................................................................. 18
  2.5 SMG Dissociation ............................................................................................................... 18
  2.6 Drug treatment ..................................................................................................................... 19
  2.7 Explant cultures .................................................................................................................... 20
  2.8 Immunohistochemistry ....................................................................................................... 20
  2.9 Quantification ....................................................................................................................... 21
  2.10 Patch clamp electrophysiology ......................................................................................... 22
  2.11 Ca^2+ imaging ..................................................................................................................... 24
2.12 Statistical analysis........................................................................................................................................25

Chapter 3 .........................................................................................................................................................26

3.1 Enhanced neurite outgrowth in a colitis model ..........................................................................................26
3.2 Superior mesenteric ganglion expresses IL-17 receptors ........................................................................26
3.3 Interleukin-17 enhances neurite outgrowth ............................................................................................30
3.4 Inhibition of Ca²⁺ currents by IL-17 ........................................................................................................34
3.5 Attenuating I_Ca causes enhanced growth .............................................................................................38
3.6 Electrophysiological and outgrowth effects of IL-17 can be reversed using an NF-κB inhibitor ..............................................................................................................................................40
3.7 Enhanced growth does not depend on glial cells ..................................................................................40
3.8 IL-17 is chemotactic for extending neurites ........................................................................................44

Chapter 4 Discussion .......................................................................................................................................49

4.1 Increased neurite outgrowth in a colitis model ........................................................................................50
4.2 Cytokines and neurite outgrowth ...........................................................................................................50
4.3 Ca²⁺ currents and IL-17-induced outgrowth ..........................................................................................52
4.4 Voltage-gated Ca²⁺ channels and outgrowth ........................................................................................53
4.5 IL-17 acts through the NF-κB pathway ..................................................................................................54
4.6 Involvement of glial cells .......................................................................................................................56
4.7 Broader significance: the SNS and IBD ...............................................................................................57
4.8 Future directions .......................................................................................................................................59
List of Figures

Figure 1. The sympathetic innervation of the GI tract .......................................................... 2
Figure 2. Schematic of neurite outgrowth quantification ......................................................... 23
Figure 3. Myeloperoxidase assay of inflammation ................................................................. 27
Figure 4. Increase in neurite outgrowth following DSS colitis .................................................. 28
Figure 5. RT-PCR showing the presence of the IL-17 receptor ................................................. 29
Figure 6. Increased neurite outgrowth after 7 days of IL-17 treatment ....................................... 31
Figure 7. Increased neurite outgrowth after 3 days of IL-17 treatment ....................................... 32
Figure 8. Concentration-response analysis of neurite growth ................................................... 33
Figure 9. Effect of IL-17 on ICa ............................................................................................. 35
Figure 10. Ratiometric Ca^{2+} imaging studies ....................................................................... 36
Figure 11. Ca^{2+} imaging of SMG growth cones ..................................................................... 37
Figure 12. Effect of conotoxin and tetrodotoxin on neurite growth .......................................... 39
Figure 13. SC-514 prevents the effects of IL-17 on ICa .............................................................. 41
Figure 14. Blockade of NF-κB signaling prevents effect of IL-17 on outgrowth ....................... 42
Figure 15. IL-17 did not cause a significant proliferation of glial cells ...................................... 43
Figure 16. Reduction in glial cell numbers after treatment with AraC ........................................ 45
Figure 17. AraC did not affect the IL-17-induced increase in outgrowth .................................... 46
Figure 18. 7 day outgrowth results in the presence of AraC ....................................................... 47
Figure 19. Directional growth of neurites towards a source of IL-17 .......................................... 48
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM</td>
<td>acetoxymethylester</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AraC</td>
<td>cytosine arabinoside</td>
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<td>ATP</td>
<td>adenosine – 5’ – triphosphate</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>Ca²⁺</td>
<td>calcium ion</td>
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<tr>
<td>[Ca²⁺]ᵢ</td>
<td>intracellular Ca²⁺ concentration</td>
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<td>CICR</td>
<td>Ca²⁺-induced Ca²⁺ release</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
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<td>CTX</td>
<td>conotoxin</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
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<td>DSS</td>
<td>dextran sulphate sodium</td>
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<tr>
<td>ENS</td>
<td>enteric nervous system</td>
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<td>ERK</td>
<td>extracellular regulated kinase</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>GI</td>
<td>gastrointestinal</td>
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<tr>
<td>HBSS</td>
<td>Hank's buffered salt solution</td>
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<tr>
<td>HuD</td>
<td>Hu protein D</td>
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<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
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<tr>
<td>IₛCa</td>
<td>voltage-gated Ca²⁺ current</td>
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<tr>
<td>IₛK</td>
<td>Iκ kinase</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IL-17R</td>
<td>interleukin-17 receptor</td>
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<tr>
<td>JNK</td>
<td>c-Jun terminal kinase</td>
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<tr>
<td>K⁺</td>
<td>potassium ion</td>
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<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
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<td>MAPK</td>
<td>mitogen associated protein kinase</td>
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<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>mV</td>
<td>millivolt</td>
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<tr>
<td>NA</td>
<td>noradrenaline</td>
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<td>NF-κB</td>
<td>nuclear factor - κB</td>
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<td>NGF</td>
<td>nerve growth factor</td>
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<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
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<td>OSM</td>
<td>oncostatin M</td>
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<tr>
<td>pA</td>
<td>picoAmpere</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>picoFarad</td>
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<td>paraformaldehyde</td>
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<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
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<tr>
<td>ROI</td>
<td>region of interest</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
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<td>SMG</td>
<td>superior mesenteric ganglion</td>
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<tr>
<td>SNAP</td>
<td>synaptosome-associated protein</td>
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<tr>
<td>SNS</td>
<td>sympathetic nervous system</td>
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<tr>
<td>SOX10</td>
<td>SRY(sex determining region-Y)-box 10</td>
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<tr>
<td>TEA</td>
<td>tetraethylammonium</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>Th</td>
<td>helper T cell</td>
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<tr>
<td>TNBS</td>
<td>trinitrobenzene sulphonic acid</td>
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<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<tr>
<td>TrkA</td>
<td>tropomyosin-related kinase A</td>
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<td>TTX</td>
<td>tetrodotoxin</td>
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<td>VGCC</td>
<td>voltage-gated Ca$^{2+}$ channel</td>
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CHAPTER 1

Introduction

Neural regulation of the functions of the gastrointestinal (GI) tract relies on the coordinated actions of several neuronal populations, including intrinsic neural circuitry unique to the gut known as the enteric nervous system (ENS) (Furness & Costa, 1987). The ENS is responsible for unique features of the GI tract; it is the only visceral structure in the body that still possesses motor reflexes when isolated from the spinal cord. Along with the nerves of the ENS, spinal afferent nerves and autonomic nerves contribute to sensation, motility, and secretion in the gut. A fine balance between of all these neuronal populations is crucial to normal GI function.

1.1 Sympathetic innervation of the gastrointestinal tract

In addition to the ENS, the gut receives efferent innervation from the parasympathetic and sympathetic branches of the autonomic nervous system. Sympathetic preganglionic neuron cell bodies residing in the central nervous system (CNS), mainly in the intermediolateral cell column of the spinal cord, project axons that synapse either in paravertebral or prevertebral ganglia outside the CNS. The sympathetic neurons that innervate the GI tract have their postganglionic cell bodies in a series of prevertebral ganglia (Furness & Costa, 1987; Szurszewski & Miller, 1994; Szurszewski et al., 2002). One important prevertebral ganglion is the superior mesenteric ganglion (SMG), which is located at the junction of the superior mesenteric artery and the abdominal aorta, and sends projections to the distal small intestine and proximal large intestine (Figure 1). This ganglion receives innervation from
Figure 1
A schematic depicting the sympathetic innervation of the GI tract. The superior mesenteric ganglion, located near the branch of the superior mesenteric artery from the abdominal aorta, contains the cell bodies of the sympathetic neurons that innervate the distal small intestine and proximal large intestine. Preganglionic neurons in the thoracolumbar region of the spinal cord send projections to the SMG via the thoracic splanchnic nerves (pale blue), where they synapse with postganglionic neurons (example highlighted in green). (Modified from Miolan and Niel, 1996)
preganglionic and postganglionic sympathetic neurons, axon collaterals of spinal afferent neurons, and from a subclass of enteric neurons termed intestinofugal neurons (Szurszewski et al., 2002). Therefore, this ganglion can be thought of as a complex integration centre, rather than a simple output relay station (Miolan & Niel, 1996). This integrative role is critical in coordination of GI function, and alterations in the properties of neurons within the ganglion may have significant functional repercussions.

Sympathetic innervation of the bowel modulates a variety of functions, including secretion, motility, and blood flow. The SNS can exert both direct and indirect control over these functions. SMG neurons can act as motor neurons, directly causing constriction of vascular smooth muscle through co-release of a purine neurotransmitter (probably adenosine triphosphate (ATP)) and noradrenaline (NA), potentiated by release of neuropeptide Y (NPY) (Lundberg et al., 1989; Westfall et al., 2002). These neurons can also have an indirect modulatory effect on GI function, acting as interneurons that synapse on enteric neurons in the submucosal and myenteric plexuses. In the guinea pig, the nerve fibres arising from sympathetic postganglionic neurons that project to the submucosal plexus contain NA and somatostatin, and lead to a reduction in secretion via α2 adrenoceptors (Shen & Surprenant, 1990). Neurons that project to the neurons of the myenteric plexus can inhibit GI motility via release of NA that binds to both pre- and post-synaptic α2 adrenoceptors (Blandizzi et al., 1993).
1.2 Neural regulation of GI function is altered during inflammation

In such disease states as inflammatory bowel disease (IBD), pathological changes in such GI functions as motility, blood flow, and secretion are common. The symptoms manifested in IBD, such as diarrhea and abdominal pain, suggest that neural regulation of these physiological functions is compromised. Inflammation profoundly affects a wide variety of neuronal functions, including the release of neurotransmitter (Jacobson et al., 1997) and neuronal excitability (Stewart et al., 2003; Lomax et al., 2005). The ganglia innervating the GI tract, including prevertebral ganglia such as the SMG, are surrounded by a capsule of connective tissue; however, unlike the paravertebral sympathetic ganglia, the blood supply to prevertebral ganglia consist of fenestrated capillaries (Jacobs, 1977; Baker et al., 1989; Szurszewski & Miller, 1994). This means that circulating macromolecules such as inflammatory cytokines have access to the neuronal cell bodies and can directly affect their properties.

Excitation-secretion coupling, a crucial step in the transmission of nerve impulses, requires calcium (Ca\(^{2+}\)) influx into the presynaptic terminal through N-type Ca\(^{2+}\) channels at the nerve terminal (Wheeler et al., 1994). Alterations in the expression, function, or gating of voltage-gated Ca\(^{2+}\) channels (VGCCs) may result in reduced neurotransmitter release and reduced effectiveness of sympathetic signaling. Pathological situations involving elevated serum cytokine levels have been shown to affect the release of sympathetic neurotransmitters in a variety of contexts. Intestinal inflammation induced by *T. spiralis* infection reduces the release of noradrenaline from sympathetic nerve terminals in the rat jejunal myenteric plexus (Swain et al., 1991). Trinitrobenzene
sulphonic acid (TNBS) colitis reduces the release of tritiated noradrenaline from myenteric mucosal preparations from both the inflamed distal colon and, importantly, from the uninflamed jejunal preparations, indicating a potential systemic effect on the sympathetic nerves (Jacobson et al., 1995). Motagally et al. (2009b) demonstrated a significant reduction in N-type Ca\(^{2+}\) currents in the SMG of mice with dextran sulphate sodium (DSS)-induced colitis. Therefore, it is possible that inflammation reduces the ability of neurons exposed to circulating cytokines to release neurotransmitter in response to depolarization. The actions of inflammatory cytokines and other factors such as oxidative stress at the nerve terminal itself may also affect the structural integrity and neurotransmission of sympathetic fibres.

1.3 Interleukin 17

1.3.1 Induction and maintenance of Th17 cells

T lymphocytes, and the pro-inflammatory cytokines they secrete, are thought to be largely responsible for the tissue damage and systemic effects of IBD. Helper T cells, or Th cells, start out as an undifferentiated progenitor population and can be induced into various differentiated phenotypes, depending on the cytokines to which they are exposed. In addition to the traditional dichotomy between Th1 and Th2 lineages, the existence of a third subset of helper T cells – Th17 – has recently been established (Bettelli et al., 2007). In the past, it was thought that Crohn’s disease was perpetuated by Th1 cells and ulcerative colitis by Th2 cells, but this paradigm has been increasingly challenged by the
discovery of the novel Th17 lineage, which seems to overlap the two illnesses (Basso et al., 2009).

Th17 cells require an independent cytokine profile from Th1 or Th2 cells and unique transcription factors in order to differentiate. Th17 cells are critical to enhancing host protection against extracellular bacteria and fungi and are implicated in the induction of autoimmune disease (Korn et al., 2007). The maintenance of this lineage requires IL-23, which is released from dendritic cells, and activates STAT3 and STAT4 pathways after binding its receptor, IL-23R, on undifferentiated Th cells (Gaffen, 2004). Once differentiated into the Th17 lineage, these cells secrete mainly interleukin 17 (IL-17) as their effector cytokine, though they can also release IL-6 and tumour necrosis factor-alpha (TNF-α) (Bettelli et al., 2007). Other cell types such as CD8+ T cells and monocytes can also be induced to secrete IL-17, resulting in further recruitment of immune cells (Xavier & Podolsky, 2007). IL-17 has an important role in such autoimmune diseases as rheumatoid arthritis and multiple sclerosis, and mounting clinical and experimental evidence points to its pivotal role in IBD (Basso et al., 2009).

1.3.2 Experimental evidence for the effects of IL-17 in the GI tract

Evidence pointing to the necessity of IL-17 in the induction and maintenance of IBD, as well as in vitro evidence of possible mechanisms for its effects, has been steadily accumulating over the past five years. In murine models of IBD, IL-17 is elevated in both DSS and TNBS colitis (Alex et al., 2009). Mice lacking the IL-17 receptor (Zhang et al., 2006) or gene (Ito et al., 2008) are significantly protected from experimental
colitis, and inhibiting the Th17 axis using antibody interference also confers protection (Hue et al., 2006). The understanding of the tissue-specific effects of this cytokine, however, is in its infancy, particularly with regards to the nervous system. Several studies have demonstrated detrimental effects of IL-17 on cultured intestinal epithelial cells (Schwartz et al., 2005), whereas others have demonstrated a cytoprotective role for IL-17 (Kinugasa et al., 2000). There have been no investigations into the direct effects of IL-17 on neuronal function, despite its being identified as a critical component of neuronal autoimmune pathologies (Hofstetter et al., 2009).

1.3.3 Clinical evidence for a role of IL-17 in IBD

Emerging clinical evidence indicates that IL-17 plays a central role in the pathogenesis of IBD. Biopsies taken from patients with active Crohn’s disease and ulcerative colitis had significantly higher levels of Th17 cells than healthy controls (Annunziato et al., 2007). Tissue and serum levels of IL-17 are elevated in IBD patients, most notably those with Crohn’s disease (Fujino et al., 2003). In a genome-wide study, certain polymorphisms in the IL-23R gene (which would reduce induction of the IL-17 pathway) conferred protection from Crohn’s disease, while others increase susceptibility (Duerr et al., 2006). In concert with in vitro evidence of its actions on intestinal tissues, these data indicate a potential for a pivotal role of this cytokine in IBD. The precise effects of IL-17 on the pathogenesis of intestinal inflammation remain to be investigated.
1.3.4 Potential significance

Although circumstantial evidence for the role of IL-17 in the pathogenesis of IBD is strong, there is little mechanistic evidence as to how IL-17 exerts its effects, particularly in the nervous system. Given this paucity of knowledge, the central role of IL-17 in inflammatory diseases, and evidence of sympathetic nervous dysfunction during IBD, the specific effects of IL-17 on sympathetic neurons remains an important area for further exploration. Previous studies have demonstrated that the inflammatory cytokines elevated during IBD, specifically TNF-α, have direct inhibitory effects on voltage-gated Ca$^{2+}$ currents in sympathetic neurons (Motagally et al., 2009a). Emerging evidence for the role of IL-17 in IBD led to the hypothesis that this novel cytokine could alter sympathetic neurophysiology. We have determined that the SMG neurons express mRNA for the IL-17 receptor using RT-PCR. I hypothesized that modulation of Ca$^{2+}$ levels in sympathetic neurons may be the mechanism of this cytokine’s effects on the SNS.

1.4 Ca$^{2+}$ currents and synaptic transmission

The delicate control of intracellular Ca$^{2+}$ levels is critical to a wide range of neuronal functions, in particular synaptic transmission. Depolarization during an action potential opens voltage-gated Ca$^{2+}$ channels at the presynaptic terminal, allowing an influx of extracellular Ca$^{2+}$ ions. This elevated [Ca$^{2+}$]$_i$ causes fusion of synaptic vesicles with the presynaptic membrane through the actions of Ca$^{2+}$-sensing proteins on the
vesicular and pre-synaptic membranes, resulting in release of neurotransmitter (Zimmerberg et al., 2006). The Ca$^{2+}$-sensing protein synaptotagmin is tethered to the synaptic vesicle and allows docking of the vesicular membrane to the protein complexes located on the presynaptic membrane (Catterall et al., 2006). These protein complexes, termed SNARE complexes, consist of syntaxin, SNAP-25, and VAMP proteins, and work in concert to allow fusion of synaptic vesicles with the presynaptic membrane (Zimmerberg et al., 2006). Together, the actions of these Ca$^{2+}$-sensitive proteins allow the coupling of an electrical event – an action potential – to the release of neurotransmitter into the synaptic cleft.

The VGCCs that mediate this excitation-secretion coupling can be separated into several subtypes, differentiated based on their voltage-dependence of activation and pharmacological sensitivities. High-voltage-activated channels include L-type, N-type, P/Q-type, and R-type Ca$^{2+}$ channels, and low-voltage activated channels include T-type channels. Previous studies have shown that inward Ca$^{2+}$ currents in SMG neurons are primarily mediated through N-type (~60%) and L-type channels (Motagally et al., 2009a). Release of neurotransmitter from these neurons is N-type channel dependent (Smith & Cunnane, 1997), and any factors affecting the influx of Ca$^{2+}$ ions through these channels would result in decreased neurotransmitter release. Although the studies were conducted at the level of the neuron cell body, reductions in I$_{Ca}$ at the cell body correlate with decreased release of neurotransmitter from varicosities in the GI tract (Motagally et al., 2009b). Therefore, any inflammation-induced alterations in Ca$^{2+}$ flux are likely to
affect sympathetic synaptic transmission, as well as other Ca\(^{2+}\)-modulated neuronal functions such as the growth of neurites.

1.5 Ca\(^{2+}\) currents and neurite outgrowth

In addition to the release of neurotransmitters, intracellular Ca\(^{2+}\) is involved in a plethora of neuronal processes critical to proper development and function. Specifically, the role of Ca\(^{2+}\) in neurite extension and growth cone guidance is an intriguing field. During development and following axotomy, neurites extending from neuronal cell bodies use various environmental cues to “pathfind” to the appropriate targets. It is clear from a large body of research that intracellular Ca\(^{2+}\) transients play a central role in this pathfinding behaviour (Bolsover, 2005; Spitzer, 2006).

Controversy remains as to whether an increase or a decrease in inward I\(_{\text{Ca}}\) is responsible for enhancing the growth of neurites. Nishiyama et al. (2003) found that a localized increase in the open probability of VGCCs underlies a positive growth cue. In contrast, the in vivo cessation of outgrowth of cerebellar Purkinje neurons occurs once they begin electrical activity, i.e. once inward current through VGCCs begins to be present (Schilling et al., 1991). A measurable rise in growth cone Ca\(^{2+}\), mediated by VGCCs, appears to underlie the collapse of growth cones when locus ceruleus neurons encounter a stop signal (Moorman & Hume, 1993). In filopodia extended by growing axons, high frequency Ca\(^{2+}\) transients inhibit neurite outgrowth, whereas low frequency of intracellular Ca\(^{2+}\) transients promotes directional growth (Gomez et al., 2001). It has been suggested that growth cone motility and elongation have distinct optima of
intracellular Ca\(^{2+}\) concentration – motility may be enhanced while elongation inhibited at a particular Ca\(^{2+}\) concentration (Kater et al., 1988). Differing spatial and temporal characteristics of elevations of [Ca\(^{2+}\)]\(_i\) (Bolsover, 2005), as well as the relative concentration of Ca\(^{2+}\) inside and outside the cell (Gomez & Spitzer, 1999), have also been posited as mechanisms to reconcile these seemingly paradoxical phenomena.

Several studies have shown that inhibiting Ca\(^{2+}\) influx through various cation channel types enhances neurite sprouting, and a partial blockade of Ca\(^{2+}\) influx provides better results than a complete one (Gomez & Zheng, 2006). A recent study implicated N-type VGCCs in the ability of the growing neurite to transduce a “stop” signal, initiated by encountering the proper target extracellular matrix (Sann et al., 2008). The authors demonstrated that blocking N-type VGCCs resulted in increased number of in vivo nerve endings in the developing Xenopus sensory apparatus. Similarly, Ryan et al. (2007) demonstrated that depolarization inhibits neurite outgrowth from descending lamprey brain neurons, and that this inhibition is due to inward current through N-type VGCCs. Other studies have implicated L-type channels in the Ca\(^{2+}\)-dependent regulation of neurite outgrowth (Redmond et al., 2002; Kulbatski et al., 2004); however, it is likely that developmental stage plays a significant role in determining which specific subtype is expressed, and at which location (Pravettoni et al., 2000).

In addition to inward flow of extracellular Ca\(^{2+}\), an important source of Ca\(^{2+}\) in the growth cone is intracellular Ca\(^{2+}\) stores, liberated by Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from the endoplasmic reticulum. Ca\(^{2+}\) release via ryanodine receptors (RyR) amplifies signals generated by VGCC opening, and seems to play a key role in transducing both
attractive and repulsive cues (Bolsover, 2005). Rat sensory neuron growth cones usually collapse when they encounter the membrane protein N135, but this effect is reversed by depletion (using caffeine) or blockade (using the RyR blocker dantrolene) of intracellular Ca^{2+} stores (Bandtlow et al., 1993). Depleting intracellular Ca^{2+} stores with thapsigargin converts the normally negative guidance cues SDF-1 and GABAB agonists into neutral ones in rat cerebellar granule cell and *Xenopus* axons, respectively (Xiang et al., 2002). Similarly, the chemoattractive effect of netrin on *Xenopus* spinal neurons in neutralized by depleting the intracellular Ca^{2+} stores (Hong et al., 2000). Thus, the ability of intracellular Ca^{2+} sources to be mobilized in response to guidance cues is necessary in the proper physiological functioning of the growth cone.

Inhibition of inward I_{Ca} would also affect the ability of cells to utilize internal Ca^{2+} stores locally in the growth cone. If IL-17 were to exert an inhibitory effect on current through VGCCs, similar to TNFα, the ability of sympathetic neurites to maintain appropriate growth responses would be altered. For example, an inability to collapse when encountering a negative guidance cue (Bandtlow et al., 1993) could result in over-stimulated outgrowth.

1.6 IL-17: A neurotrophic cytokine?

The possibility that inflammatory cytokines such as IL-17 can have multiple roles – exerting both tissue-damaging and growth-promoting effects – has precedents in the literature. Several cytokines with established neurotrophic effects have been grouped into the “neuropoietic” family of cytokines (Bauer et al., 2007). The neuropoietic family
includes the cytokines ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM) and transforming growth factor (TGF)-β, as well as several interleukins, including IL-6 and IL-11 (Marz et al., 1998). The presence of cytokines is neuroprotective and supportive of axonal regeneration in many contexts. Activated macrophages injected into the site of spinal cord injury significantly enhance functional recovery in rats (Rapalino et al., 1998). Transgenic mice overexpressing IL-6 and the soluble IL-6 receptor show markedly accelerated nerve regeneration, and nerve regeneration is also enhanced by soluble factors secreted by macrophages (Otten et al., 2000; Yin et al., 2006). Blocking the action of several inflammatory cytokines, including IL-3, IL-1β, and IL-6, results in significant inhibition of the targeted growth of sympathetic neurites (Kannan et al., 1996). There is ample evidence of upregulation of neurotrophin expression during inflammation of the lungs (Virchow et al., 1998), skin (Hendrix & Peters, 2007), and intervertebral discs (Purmessur et al., 2008). Indeed, the line between cytokine and neurotrophin is being blurred in both directions; a recent paper argued for the canonical neurotrophin NGF to be classified as a cytokine based on several lines of evidence, including its role in activating immune cells (Bonini et al., 2003). The relationship between inflammation and growth factor expression is evidently a complex one.

As well as the general overlap between cytokines and neurotrophic factors, IL-17 in particular has structural similarities to the canonical neurotrophins. There are six members of the IL-17 family: IL-17A (IL-17) through F. Secreted IL-17F has been demonstrated to adopt a cystine knot conformation characteristic of a variety of growth
factors, such as NGF, TGF-β, and platelet-derived growth factor (PDGF) (Moseley et al., 2003). IL-17A and IL-17F bind the same receptor and share the highest degree of homology (50%) of all the IL-17 family members. They are mapped to the same location of the same chromosome, 6p12 (Moseley et al., 2003); therefore structural similarities between IL-17A and IL-17F are likely, particularly with respect to receptor binding. There are five highly conserved cystines across the IL-17 family, four of which are responsible for formation of the cystine knot; therefore it is plausible that all members, especially IL-17A which is closest to IL-17F, exhibit similar crystal structures. The only difference between the cystine knot of IL-17F and other neurotrophins such as NGF is that the former uses 6 cystines instead of 4. IL-17 is secreted as a dimer, resulting in the molecule adopting a surface pocket in an identical location to a similar one on NGF that is responsible for NGF binding its receptor, TrkA (Moseley et al., 2003). In summary, structural evidence suggests that IL-17 adopts a similar structure and receptor binding domain to several known growth factors.

1.7 Inflammation and increased nerve sprouting

If indeed IL-17 is acting as both an inflammatory mediator and a neurotrophin, what is the relevance to sympathetic neural architecture and function in vivo? Several studies have demonstrated an increase in sympathetic nerve fibre density in states of inflammation. Following myocardial infarction, there is a marked sympathetic hyperinnervation surrounding the inflamed area (Hasan et al., 2006). During interstitial cystitis, an inflammation of the lining of the bladder, there is a similar increase in the
ingrowth of sympathetic nerve fibres (Peeker et al., 2000). In regards to the GI tract, a consensus has yet to be reached. Straub and colleagues (2008) showed that the number of tyrosine hydroxylase-positive fibres in the inflamed colon was reduced relative to control; however, these samples were taken from sites of active inflammation where damage may have been ongoing. In contrast, in the DSS and TNBS models of murine colitis, an increase in tyrosine hydroxylase and dopamine beta-hydroxylase immunoreactivity was shown in the colonic mucosa and lamina propria (Bai et al., 2009). An increase in the number and diameter of nerve fibres (immunopositive for the neurofilament synaptophysin and the NGF receptor) was observed in the ileum and colon of patients with Crohn’s disease (Strobach et al., 1990). Finally, Birch et al. (2008) demonstrated an increased density of sympathetic innervation in inflamed arteries and veins of human patients.

Whether increased nerve fibre density following inflammation is adaptive or pathological is a difficult question to answer. In the gut, sympathetic nerves exert a tonic anti-inflammatory effect through the actions of NA on β-2 and β-3 adrenoceptors expressed on immune cells (Lomax, 2008). It may be that the observed hyperinnervation is an attempt to re-establish neuronal immunomodulation following an inflammatory insult. Conversely, an ingrowth of sympathetic nerves may be maladaptive and have a role in continuing the pathologies of IBD. Sprouting of sympathetic fibres into sensory ganglia underlies neuropathic pain (Zhang et al., 2004). Perhaps the continuing visceral hypersensitivity and abdominal pain that plagues IBD patients is a result of hyperactive sensory nerves under the inappropriate control of wayward sympathetic axons.
1.8 Unresolved issues

To date, there have been no experiments investigating the effects of IL-17 on neuronal function nor has there been any elucidation of the signaling pathway(s) responsible for any actions exerted on neurons. Several downstream effector pathways have been proposed for IL-17 in the immune system, including JNK (Wuyts et al., 2005), p38 and ERK/MAPK (Laan et al., 2001), and NF-κB (Kehlen et al., 2001). Most of the elucidation of signaling pathways was conducted in immune cells; it is unclear which precise pathways are the most important and which are activated in neurons. Alteration in the sympathetic regulation of the gastrointestinal tract during inflammation is an important but understudied area, and a possible role of IL-17 in this phenomenon remains to be explored.

1.9 Hypothesis

The importance of IL-17 in the pathogenesis of IBD, a disease which involves dysregulated neural function, as well as the experimental precedents for cytokines affecting neuroplasticity led us to ask the question: can IL-17 act as a neurotrophic factor? If so, what are the mechanism and the intracellular signaling pathway responsible? **We hypothesized that 1) IL-17 causes increased outgrowth of sympathetic neurites and 2) the mechanism of action is an alteration in inward $I_{Ca}$, an important determinant of neurite architecture.**
Chapter 2

Methods

2.1 Animal care

All animal care protocols were developed in accordance with Queen’s University Animal Care Committee regulations and followed the Guidelines of the Canadian Council of Animal Care. Adult male CD1 mice weighing between 25-35 grams were purchased from Charles River (Saint Constant, QC) and maintained on a 12-hour light-dark cycle, with access to standard lab chow and tap water ad libitum.

2.2 IBD model

For the induction of colitis, 5% DSS was included in drinking water for 5 days, followed by 2 days of normal drinking water. The mice were monitored for signs of dehydration, bloody stools and diarrhea. Age-matched control mice received normal drinking water for the duration of the study.

2.3 Tissue collection

Following deep anesthesia by an overdose of inhaled isoflurane, mice were killed by cervical dislocation. A laparotomy was performed, exposing the peritoneal cavity and internal organs. The superior mesenteric ganglion (SMG) was isolated from just rostral to the left renal artery, dissected free and cleaned of all visible fat under a dissecting microscope, then quickly transferred to a vial of pre-warmed Hank’s buffered salt solution (HBSS). In mice with DSS-induced colitis, a sample of proximal colon was collected, cleaned of its contents, and flash-frozen in liquid nitrogen for subsequent
myeloperoxidase (MPO) assay (a measure of neutrophil infiltration). MPO activity is expressed in units per gram of tissue collected, with one MPO unit representing the degradation of 1 μmol of hydrogen peroxide in 1 minute.

2.4 Reverse transcriptase PCR

RNA was extracted using the Trizol method (Invitrogen) from whole SMG of control mice. Expression of IL-17 receptor (IL-17R) A, B, C and D was detected using specific primers (Table 1). cDNA was reverse transcribed from the collected RNA using Expand reverse transcriptase (Roche) and Oligo (dT) Primer (Invitrogen), and amplified by PCR for 30 cycles.

**TABLE 1 – Mouse primers used for RT-PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
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<tr>
<td>IL-17RA</td>
<td>CCATCAGCGAGCTAATGTCA</td>
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<tr>
<td></td>
<td>AATGGCGATGAGTGTGATGA</td>
</tr>
<tr>
<td>NM_008359</td>
<td>217 bp</td>
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<tr>
<td>IL-17RB</td>
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<tr>
<td></td>
<td>GAAAGGACGTCTTCGTGCTC</td>
</tr>
<tr>
<td>NM_019583</td>
<td>208 bp</td>
</tr>
<tr>
<td>IL-17RC</td>
<td>AGATGCTCTGTCTCCTGGTC</td>
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<tr>
<td></td>
<td>CGCAATCTGTCTTCGTGGA</td>
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<tr>
<td>NM_134159</td>
<td>243 bp</td>
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<tr>
<td>IL-17RD</td>
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<td></td>
<td>GTGCTGGCCTTGAAGAAGAC</td>
</tr>
<tr>
<td>NM_134437</td>
<td>220 bp</td>
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</table>

2.5 SMG Dissociation

For patch clamping, Ca²⁺-imaging and most neurite outgrowth experiments, the SMG was dissociated to yield single neurons as follows. The ganglion was washed 3 times with HBSS warmed to 37°C, and subsequently was replaced by collagenase
solution (2.5 mg/mL) with BSA (6 mg/mL) in HBSS. The tissue was incubated at 37°C for 25 minutes, and subsequently washed a further 3 times with warmed HBSS. Trypsin solution (1 mg/mL) with BSA was then added for 15 minutes, and was inactivated by the addition of media containing 10% fetal bovine serum (FBS). The media-trypsin solution mixture was removed and replaced with Leibowitz’s (L-15) media supplemented with 24 mM NaHCO3, 10 % FBS, 38 mM D-glucose, 5000 IU penicillin/streptomycin, and 50 ng/ml NGF. The SMG was mechanically dissociated into a suspension of single cells using a series of fire-polished Pasteur pipettes of decreasing diameters. 100-200 μL of cells were plated onto sterilized, laminin (10 ug/mL)-coated glass coverslips in a 24-well plate and allowed to adhere for 2.5 hours, at which time media was added to total 1 mL per well.

2.6 Drug treatment

Recombinant mouse interleukin-17 (Peprotech, Rocky Hill, NJ, USA) was added to the culture medium or incorporated into collagen gels at concentrations of 100 pg/mL, 1 ng/mL or 10 ng/mL, corresponding to physiologically relevant concentrations. Unless otherwise stated, the intermediate dosage (1 ng/mL) was used for experimental procedures. For NF-κB inhibition studies, 20 μM SC-514 (Sigma, St. Louis, MO, USA) was added to the wells 2 hours prior to addition of IL-17, and was re-added each day. Tetrodotoxin (TTX, Tocris) or ω-Conotoxin GVIA (CTX, Sigma) was added to short-term culture dishes at concentrations of 300nM and 30 nM, respectively. To create enriched neuronal cultures with little to no glia, 5 μM cytosine arabinoside (AraC, Sigma; Besirli et al., 2003) was added after 24 hours in vitro. For long-term culture experiments,
fresh media was added at Day 3 and Day 6 in vitro, including the appropriate concentration of any cytokines, inhibitors and/or chemicals.

2.7 Explant cultures

Collagen gels were formed using 8 parts acidified rat tail collagen (Roche) reconstituted with 1 part 10x concentrated DMEM and 1 part 10x concentrated HBSS, and diluted to 2 mg/mL with DMEM containing 5% FBS (Barrett et al., 2005). NGF was incorporated into the gels at a final concentration of 50 ng/mL. SMG were collected as described above, washed 3 times in warmed HBSS, and placed in collagenase solution for 20 minutes to facilitate breakdown of the ganglion capsule. Ganglia were then washed, cut into 4 equal-sized pieces under a dissecting microscope, and each piece was embedded into the semi-solidified collagen gel using fine forceps. For directional growth assays, Cibicron agarose beads (Sigma) were impregnated with either NGF (100 ng/mL) or IL-17 (10 ng/mL), dissolved in HBSS. Control beads were incubated with HBSS alone. A single bead was placed 800-900 μm from the ganglion explant. Bead position was marked prior to immunohistochemical processing.

2.8 Immunohistochemistry

Tissues were fixed using 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer. Dissociated cells were fixed for 10 minutes; collagen gels containing explants were fixed overnight. Following removal of the fixative and four washes in PBS, the tissues were blocked for 1 hour in either 5% normal goat serum or 5% normal horse serum in PBS with 0.1% Tween (depending on the species in which the secondary antibody was raised). Primary antibodies to SNAP-25, HuD, SOX10, or β-3 tubulin were
then added overnight at 4°C at various concentrations (see Table 2). Primary antiserum was removed, tissues were washed a further three times in PBS, and an appropriate secondary antibody (see Table 3) was added for one hour at room temperature. After a further 3 washes in PBS, coverslips were mounted with buffered glycerol and visualized using an epifluorescence microscope (Olympus BX51). Photos were taken using a Coolsnap CCD camera and ImagePro software.

### TABLE 2 – Primary Antibodies

<table>
<thead>
<tr>
<th>Reactivity</th>
<th>Host</th>
<th>Source</th>
<th>Concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuD</td>
<td>Mouse</td>
<td>Invitrogen</td>
<td>1:500</td>
<td>(Antunes et al., 2000)</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Rabbit</td>
<td>Invitrogen</td>
<td>1:4000</td>
<td>(Geddes et al., 1990)</td>
</tr>
<tr>
<td>SOX10</td>
<td>Goat</td>
<td>Santa Cruz</td>
<td>1:500</td>
<td>(Liu et al., 2002)</td>
</tr>
<tr>
<td>β-3 tubulin</td>
<td>Rabbit</td>
<td>Sigma</td>
<td>1:500</td>
<td>(Leiss et al., 1988)</td>
</tr>
</tbody>
</table>

### TABLE 3 – Secondary Antibodies

<table>
<thead>
<tr>
<th>Reactivity</th>
<th>Host</th>
<th>Source</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Goat</td>
<td>Invitrogen</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Goat</td>
<td>Invitrogen</td>
<td>1:2000 for SNAP&lt;br&gt;1:1000 for β-3 tubulin</td>
</tr>
</tbody>
</table>

### 2.9 Quantification

For HuD / SNAP-25-stained coverslips, every third field of view in the horizontal and vertical axes using the 40x lens was analyzed. The number of HuD-positive neurons in the entire field, and the number of SNAP-25-positive neurites crossing either the vertical or horizontal midline of the field of view were determined. For real time
outgrowth assays, the number of neurons, the number of neurites, the number of branch points, and the number of neurons with neurites on half of each coverslip were recorded. A neurite was included for analysis if it was greater than one cell body in length. For β-3-tubulin-stained explants, regions of interest (ROI) adjacent to each ganglion were identified on thresholded black-and-white images. An “experimental” ROI 750 x 750 pixels in size was selected on the side of the explant facing the bead. Using ImageJ software (rsbweb.nih.gov/ij/), the total positive immunofluorescence within the “experimental” ROI was calculated and compared to a “control” ROI of the same size on the opposite side to the bead (See Figure 2).

2.10 Patch clamp electrophysiology

Dissociated SMG neurons were cultured overnight in the presence or absence of 1 ng/mL IL-17 and/or other drugs. All electrophysiological recordings were carried out at room temperature (21-22°C). To record current through VGCCs (carried by Ba²⁺), coverslips of dissociated neurons were superfused with an external solution of the following composition (mM): 140 tetraethylammonium (TEA)-Cl, 2 MgCl₂, 5 BaCl₂, 10 glucose, 10 HEPES, brought to pH 7.4 with TEA-OH. Fire-polished glass pipettes with resistances between 3 and 8 MΩ were back-filled with internal solution containing (mM): 120 CsCl, 1 MgCl₂, 4 MgATP, 0.3 NaGTP, 10 EGTA, 10 HEPES, and 50 ug/mL amphotericin B, brought to pH 7.2 with CsOH. Fire-polished glass pipettes were lowered onto cells visualized with a phase contrast microscope, and once a seal was established (1GΩ or greater) and perforation had taken place (access resistance less than 20 MΩ), the
Figure 2
Schematic of optical quantification method for ganglion explant cultures. Relative immunofluorescence contained in the red, “experimental” and the yellow, “control” boxes was compared using thresholded images in ImageJ software. Regions of interest were positioned along the line connecting the agarose bead to the centre of the ganglion explant.
series resistance and cell membrane capacitance were compensated at least 80%. Voltage clamp protocols were then applied using an Axopatch 200B amplifier (MDS Analytical Technologies, Mississauga, ON, Canada), stepping from a holding potential of -90 mV to command potentials from -70 mV increasing in 5 mV increments to a maximum of +50 mV. The voltage dependence of inactivation of the Ca\textsuperscript{2+} currents was measured using a protocol stepping from a series of prepulses from -70 mV to +30 mV for 500 ms to 0 mV for 150 ms, to elicit a maximal inward current after the prepulse step. Data were acquired using a Digidata 1440A analogue-to-digital converter and pClamp 10.1 software (MDS Analytical Technologies). Current recordings were normalized to cell capacitance and expressed as current density values.

2.11 Ca\textsuperscript{2+} imaging

To optically measure [Ca\textsuperscript{2+}], dissociated SMG neurons were incubated with the fluorescent Ca\textsuperscript{2+}-sensing dye FURA-2 AM (5 \(\mu\)M) for 30-45 minutes at 37\(^\circ\)C. To wash off any excess and allow for intracellular de-esterification of the dye, coverslips were allowed to acclimatize to room temperature for 10 minutes, then rinsed with HEPES buffer containing (mM): 140 NaCl, 5 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES, 10 glucose, pH adjusted to 7.4 with NaOH for 20-30 minutes prior to imaging. Coverslips were continuously superfused with the HEPES solution throughout the experiment. ROI were defined either within the cell body of the neuron, or within the growth cone for experiments on Day 2. Growth cones were identified visually by their fan-like morphology (Ibarretxe et al., 2007). The cells were illuminated at 340 nm and 380 nm once per second using a DeltaRamV high speed random access monochromater (Photon
Technology International). Images were recorded using a Photometries Cascade 512B CCD camera and ImageMaster 5.0 software. The ratio of fluorescence at 340 nm (f340) versus 380 nm (f380) excitation was calculated and used to estimate \([\text{Ca}^{2+}]_i\).

Depolarization-induced \(\text{Ca}^{2+}\) entry, evoked by superfusion of high \(K^+\) external solution of the following composition (mM): 105 NaCl, 40 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 10 glucose, pH adjusted to 7.4 with NaOH, was compared under control and experimental conditions.

### 2.12 Statistical analysis

Statistical analyses and graphs were generated using Prism software. Current-voltage relationships were analyzed using Clampfit, Version 10 (MDS Analytical Technologies). Population data were analyzed by Mann-Whitney tests for parametric data, such as neurite counts, and by Kruskal-Wallis tests for non-parametric data, such as comparing percent changes. A two-way ANOVA was utilized when analyzing current-voltage relationships, with Bonferroni’s post hoc test to determine significant differences between the group means. Data are represented with error bars indicating the standard error of the mean (SEM). Statistical significance was considered to be a P value of <0.05.
Chapter 3
Results

3.1 Enhanced neurite outgrowth in a colitis model

Our initial studies examined whether experimental colitis enhanced neurite outgrowth in the SMG. Neurons dissociated from the SMG of mice with DSS-induced colitis were initially compared with neurons from healthy controls after 3 days in vitro. The level of inflammation was confirmed using the MPO assay, and all DSS mice used were found to have significantly increased MPO activity relative to the established average for healthy controls in our lab (Figure 3). The percentage of neurons with neurites was significantly increased relative to control (65.2 ± 4.3 for DSS versus 40.7 ± 3.3 for control, P<0.01, Mann-Whitney test. Figure 4). In addition, the number of neurites per neuron was markedly enhanced during experimental colitis (1.4 ± 0.1 for DSS compared with 0.84 ± 0.08 for control, P<0.01, Mann-Whitney test).

3.2 Superior mesenteric ganglion expresses IL-17 receptors

Using reverse transcriptase PCR, primers for four different subtypes of the interleukin-17 receptor were amplified. Messenger RNA for IL-17 receptor A (IL-17RA), B, C and D subtypes were present in the SMG (Figure 5), confirming previous studies, which found the receptor to be ubiquitously expressed (Ge & You, 2008;Louten et al., 2009).
Figure 3
Myeloperoxidase (MPO) activity from a standard sample of control mice and from mice with DSS-induced colitis. (N = 6 control, 4 DSS; *** P<0.0001, unpaired Student’s t-test.) These experiments were conducted by Ms. Shadia Neshat.
Figure 4
SMG neurons dissociated from mice with DSS-induced colitis exhibited a significantly higher ratio of neurites to neurons (A) and percentage of cells with neurites (B) on Day 3. (N=9 coverslips per group; ** P<0.01, Mann-Whitney test).
Reverse transcriptase PCR revealed that all four subtypes of the IL-17 receptor were present in the SMG. (These experiments were carried out by Ms. Shadia Neshat).
3.3 Interleukin-17 enhances neurite outgrowth

We examined the effect of IL-17 on sympathetic neurites using two different assays of neurite outgrowth at 3-day and 7-day timepoints. In the 3-day culture model, visual scoring by phase contrast microscopy was utilized to gain insight into the initial stages of neurite growth from dissociated SMG neurons. In the 7-day culture model, immunohistochemistry was performed and the ratio of neurites to neurons was quantified using fluorescence microscopy. Significantly increased neurite growth was seen in both the 3- and 7-day culture models, as shown in Figures 6 and 7. IL-17 appeared to cause a rapid increase in neurite growth at Day 2, followed by a leveling off and steady increase to Day 3 and on to Day 7 (Figures 6 and 7). After 7 days *in vitro*, the ratio of neurites to neurons was markedly increased; $3.8 \pm 0.9$ for control compared with $17.3 \pm 2.6$ for IL-17-treated coverslips ($P<0.01$, Mann-Whitney test).

Analyzing cells on a daily basis after 1, 2 or 3 days in culture, the ratio of neurites to neurons was significantly increased relative to control at Days 2 and 3 ($P<0.05$, Mann-Whitney test). The percentage of cells with neurites was also significantly enhanced by treatment with the cytokine ($63.4 \pm 7.0$ for IL-17 versus $40.7 \pm 3.3$ for control, $P<0.05$, Mann-Whitney test). A concentration-response analysis revealed that a lower concentration of IL-17 (100 pg/mL) produced a slightly lower level of enhanced neurite outgrowth parameters (Figure 8), and a higher concentration (10 ng/mL) yielded a qualitative increase relative to the standard 1 ng/mL concentration.
Figure 6
A) Representative images of control (left) and IL-17 treated (right) cultures at Day 7 in vitro. Cultures were fixed and immunostained for HuD (a neuronal marker, green) and SNAP-25 (an axonal marker, red). B) Quantification of neurons, neurites, and the ratio of neurites to neurons at Day 7. IL-17 treatment did not significantly alter the number of neurons per coverslip, caused a marked increase in the number of neurites per coverslip, and caused a significant increase in the ratio of neurites to neurons (P<0.001, Mann-Whitney test). N=8 coverslips per treatment group.
Figure 7
The effect of IL-17 on neurite outgrowth in 3-day culture. A) Cultures incubated in 1 ng/mL IL-17 had an average of 1.2 ±0.1 neurites per neuron at Day 2 and 1.3 ± 0.1 at Day 3, relative to 0.70 ± 0.1 and 0.85 ± 0.09 for control at Days 2 and 3, respectively. (P<0.05, Mann-Whitney test at Day 3). B) The percentage of cells with neurites was 63.4 ± 7.0 for IL-17 versus 40.7 ± 3.3 for control. (P<0.05, Mann-Whitney test). N = 6 coverslips per treatment group.
Figure 8
Concentration-response analysis of the number of neurites per neuron and the percentage of cells with neurites at Days 2 and 3 in vitro. (N = 3 or more coverslips per treatment group; * P<0.05, Mann-Whitney test)
3.4 Inhibition of Ca$^{2+}$ currents by IL-17

An important mechanism of neurite outgrowth regulation is the control of intracellular Ca$^{2+}$ levels (Bolsover, 2005; Spitzer, 2006). Electrophysiological studies were undertaken to determine whether IL-17 caused any alterations in I$_{Ca}$. Overnight incubation with 1 ng/mL IL-17 significantly attenuated inward voltage-gated Ca$^{2+}$ current, as measured by voltage clamp electrophysiology. Current inhibition reached statistical significance (P<0.05, two-way ANOVA with Bonferroni’s post hoc test) between -10 to +10 mV (Figure 9). The peak I$_{Ca}$ amplitude was reduced by approximately 45% from 37.6 ± 5.6 pA/pF in controls to 20.6 ± 4.4 pA/pF in IL-17-treated cells (P<0.05, unpaired t-test with Welch’s correction). Intracellular Ca$^{2+}$ imaging revealed similar results; the peak f340:f380 increase as a percent of baseline during depolarization with 40 mM extracellular K$^+$ was significantly reduced by overnight IL-17 treatment (Figure 10, P<0.0001, Mann-Whitney test). These measurements, initially investigated in the neuronal cell body for ease of data collection, were subsequently replicated in the growth cones. ROI taken from growth cones of IL-17-treated neurons at Day 2 in vitro had significantly lower percent [Ca$^{2+}$], increases relative to control (Figure 11, P<0.05, Mann-Whitney test). Day 2 was chosen for several reasons; outgrowth analysis had revealed that Day 2 comprised the maximal rate of outgrowth, and cell and neurite density was optimal for visualization at this time.
Figure 9
A) Sample traces of $I_{Ca}$ evoked by the voltage clamp protocol depicted. Population data are graphed as a current-voltage relationship (B) and as the peak inward $I_{Ca}$ (C). Treatment with 1 ng/mL IL-17 significantly reduced $I_{Ca}$ between -10 and 10 mV, and reduced the peak inward $I_{Ca}$ from $37.6 \pm 5.6$ pA/pF to $20.6 \pm 4.4$ pA/pF. (N = 21 control cells, 13 IL-17. * P<0.05).
Figure 10
Representative Ca$^{2+}$-imaging traces (A) and population data (B) showing a significant decrease in the peak intracellular Ca$^{2+}$ as a percent of baseline when cells were depolarized with 40 mM K$^+$ solution. (N=34 cells per treatment group; *** P<0.0001, Mann-Whitney test)
Two-day treatment with IL-17 significantly attenuated the percentage increase in f340:380 ratio in the growth cones of SMG neurites (N=8 cells for control, 10 cells for IL-17; * P<0.05, Mann-Whitney test).
3.5 Attenuating ICa causes enhanced growth

Given our initial electrophysiological and morphological data, and previous research in the field (Gomez and Spitzer, 1999; Ryan et al., 2007), we theorized that ICa inhibition was responsible for the enhanced neurite growth with IL-17 treatment. To reproduce the inhibition of inward ICa, we used a concentration of ω-conotoxin GVIA that would partly inhibit the current through the N-type VGCC (30 nM). Using this approach, we were able to reproduce the enhanced neurite outgrowth seen with IL-17 treatment. At Day 3, the percentage of cells with neurites and the number of neurites per neuron significantly increased relative to control (percent with neurites: 57.3 ± 2.9 versus 36.3 ± 2.4; neurites per neuron: 1.1 ± 0.08 versus 0.69 ± 0.09, Figure 12, P<0.01, two-way ANOVA with Bonferroni’s post hoc test).

It has been suggested that direct interactions of N-type VGCCs with extracellular matrix components can alter neurite branching (Sann et al., 2008). Therefore, to recapitulate the reduction in ICa without directly affecting the N-type channels, tetrodotoxin (TTX, 300 nM) was used to abolish voltage-dependent inward Ca\(^{2+}\) flux. Interestingly, similar results to the conotoxin treatment were observed; the ratio of neurites to neurons was significantly increased at Day 3, and the percentage of cells with neurites was also significantly upregulated at Days 2 and 3 (P<0.001, two-way ANOVA with Bonferroni’s post hoc test).
Figure 12
The effect of tetrodotoxin (TTX) and conotoxin (CTX) on the ratio of neurites to neurons (A) and the percentage of cells with neurites (B). * = P<0.05 for both TTX and CTX (relative to control; two-way ANOVA with Bonferroni’s post hoc test); # = P<0.05 for TTX relative to control. N=6 coverslips per treatment group.
3.6 Electrophysiological and outgrowth effects of IL-17 can be reversed using an NF-κB inhibitor

The nuclear factor κB (NF-κB) pathway plays a key role in the induction of many inflammatory signaling cascades. Using SC-514, an inhibitor of the Iκ kinase step in the NF-κB activation pathway, neurite outgrowth and Ca\(^{2+}\) current inhibition were investigated. Neurons pre-incubated in SC-514 prior to addition of the cytokine showed a near-total prevention of the I\(_{Ca}\) inhibition caused by IL-17, as measured by voltage clamp electrophysiology (Figure 13). Ca\(^{2+}\) imaging analysis showed similar results; cultures pre-treated with SC-514 had percent increases in f340:f380 that were significantly increased relative to IL-17 treatment alone (Mann-Whitney test; Figure 13). Inhibition of NF-κB also mitigated the effects of IL-17 on neurite outgrowth, using the 3-day culture model. When pre-treated with the inhibitor, IL-17 did not significantly increase the ratio of neurites to neurons or the percentage of cells with neurites relative to control (P>0.05, Mann-Whitney test. See Figure 14).

3.7 Enhanced growth does not depend on glial cells

In addition to neurons, the SMG contains glia that may participate in the effect of cytokine treatment. Initial experiments determined the effect of IL-17, if any, on the abundance of glial cells in our culture model. Treatment with IL-17 did not significantly alter the proliferation of glial cells (Figure 15). Using 5 μM cytosine arabinoside (AraC), an inhibitor of glial proliferation (see reference in Methods), we were able to deplete the
Figure 13
(A) IV curve depicting the effect of blocking the NF-κB pathway on I\textsubscript{Ca} inhibition by IL-17. With SC-514 pre-treatment, control and experimental values were not significantly different (N=21 for control, 13 for IL-17, 12 for SC-514; P>0.05 between control and SC-514, two-way ANOVA. P<0.05 between control and IL-17 from -10 to +10 mV, see Figure 9). (B) The inhibition of peak inward current density is summarized in a bar graph (P<0.05, unpaired Student’s t-test). (C) Similar results were seen using Ca\textsuperscript{2+}-imaging; the peak f\textsubscript{340}:f\textsubscript{380} ratio was significantly higher when cultures were pre-treated with 20 µM SC-514, as compared to IL-17 alone (N=34 cells per treatment group; P<0.05, Mann-Whitney test).
Figure 14
Pre-treatment with the IκK inhibitor SC-514 prevented the enhanced outgrowth caused by IL-17. In SC-514-treated coverslips, both the ratio of neurites to neurons (A) and the percentage of cells with neurites (B) was not significantly different from control (* P>0.05, Mann-Whitney test).
**Figure 15**
The effect of IL-17 on glial cell proliferation. IL-17 incubation did not significantly alter the number of glial cells per coverslip, as assessed by SOX10 immunostaining and quantification (A). Representative micrographs show nuclear SOX10 immunoreactivity in Control (B) and IL-17-treated coverslips (C) at day 7 (N=5, P>0.05, unpaired Student’s t-test).
glial population by 65% at Day 3 and 89% at Day 7 (Figure 16). Using the 3-day culture model, IL-17 in the presence of AraC still significantly increased the percentage of neurites with neurons at Day 3, as well as the number of neurites per neuron at Days 2 and 3. At Day 2, IL-17 significantly increased the number of neurites per neuron relative to control (0.57 ± 0.10 for control vs. 0.95 ± 0.14 for IL-17, P<0.05, Kruskal-Wallis test). By Day 3, the number of neurites per neuron (0.66 ± 0.07 for control vs.1.6 ± 0.20 for IL-17, P < 0.001, Kruskal-Wallis test) and the percentage of cells with neurites (42.8 ± 3.6 for control vs. 80.1 ± 7.8, P < 0.01, Kruskal-Wallis test) were significantly increased by IL-17, similar to the results with a full glial cell population (see Figure 17). Using the 7-day culture model and immunohistochemical analysis, there was a significant increase in the ratio of neurites to neurons when cultures were depleted of glia, again similar to normal culture conditions (Figure 18. Control: 7.0 ± 0.9, IL-17: 14.6 ± 3.8, P<0.05, Mann-Whitney test).

3.8 IL-17 is chemotactic for extending neurites

Using SMG explants seeded onto collagen gels, the growth of neurites towards an IL-17-impregnated agarose bead was examined. Directional growth towards an IL-17 bead was markedly increased relative to an HBSS-impregnated negative control bead (Figure 19), as measured by average immunofluorescence (see Methods), and was intermediate relative to an NGF-impregnated positive control bead.
Figure 16
Treatment with the gliotoxin AraC (5 μM) significantly reduced the number of glial cells per coverslip at 3 days (A) and 7 days in vitro (B) (** P<0.0001, unpaired Student’s t-test). N=4 or more coverslips per group. Representative fluorescence microscopy images are shown for each timepoint.
Figure 17
When the glial cell population was depleted with 5 μM AraC, IL-17 still exerted a significant neurotrophic effect on the number of neurites per neuron (A) and the percentage of cells with neurites (B). (N=7 coverslips per treatment group, * P<0.05, Kruskal-Wallis test)
Figure 18
After 7 days in vitro in the presence of the glial cell inhibitor AraC at 5 μM, 1 ng/mL IL-17 caused a significant increase in the ratio of neurites to neurons (C) but did not significantly alter the number of neurons (A) or neurites (B) per coverslip (N=11 coverslips, * P<0.05, Mann-Whitney test).
**Figure 19**
Immunohistochemical analysis of SMG explants revealed enhanced neurite outgrowth in the direction of an IL-17-impregnated bead relative to control (N = 3 per group). Bead direction is marked with a green arrow on the representative micrographs (Control: HBSS-impregnated bead; IL-17: IL-17-impregnated bead; NGF: NGF-impregnated bead). The bar graph summarizes the ratio of fluorescence from a region of interest on the side closest to the bead relative to the opposite side.
Chapter 4  
Discussion

The ability of pro-inflammatory cytokines to modulate the structure and function of neurons is a fascinating and rapidly evolving field, and provides a unique link in understanding the etiopathology of chronic inflammatory diseases. In the present study, the novel cytokine IL-17 increased neurite outgrowth from sympathetic neurons in a concentration-dependent and chemoattractive manner. Sympathetic neurons dissociated from the SMG, the ganglion providing much of the intestinal sympathetic innervation, exhibited significantly increased neurite outgrowth after incubation with IL-17 for two to seven days. With IL-17 treatment, a greater percentage of cells exhibited neurites and the number of neurites per neuron was significantly increased. Depletion of the cultures of glia using cytosine arabinoside did not alter the relative increase in outgrowth, suggesting that IL-17 enhanced neurite outgrowth via a direct effect on the neurons themselves.

In an effort to discern the underlying mechanism for this effect, the intracellular Ca\(^{2+}\) dynamics in IL-17-treated cultures were examined using electrophysiology and ratiometric Ca\(^{2+}\) imaging. The link between inward Ca\(^{2+}\) flux and regulation of neurite outgrowth is well-established (Bolsover, 2005; Spitzer, 2006), and it appears that in many systems, a rise in intracellular Ca\(^{2+}\) transduces a stop signal. In accordance with this model, treatment with IL-17 inhibited inward Ca\(^{2+}\) currents through VGCCs in cell bodies and growth cones. Interestingly, incubation with the specific N-type VGCC blocker \(\omega\)-conotoxin-GVIA increased the growth of SMG neurites to a similar level to
IL-17 incubation. Both the alterations in inward $I_{Ca}$ and the increased neurite outgrowth were prevented by inhibition of the NF-κB pathway using SC-514. This study represents the first demonstration that IL-17 exerts a neurotrophic effect, and that this effect occurs via the NF-κB pathway.

4.1 Increased neurite outgrowth in a colitis model

In an initial group of experiments, dissociated sympathetic neurons taken from mice with experimental colitis were examined for differences in the growth and morphology of neurites. At Day 3, there was a significant increase in the percentage of cells with neurites and the number of neurites each neuron sprouted in the DSS group. An association between GI inflammation and increased neurite sprouting seemed to be present, and indicated a possibility that inflammatory cytokines could play a role in modulating the properties and morphology of sympathetic neurons. In view of these findings, and the known upregulation of IL-17 expression during experimental and clinical colitis (Annunziato et al., 2007; Alex et al., 2009), specific studies were undertaken to dissect the contribution of IL-17 to the observed effects.

4.2 Cytokines and neurite outgrowth

An expanding body of knowledge, including the present study, supports the idea that inflammation – specifically, the direct actions of inflammatory cytokines – can modulate neuronal structure and function. IL-17 treatment enhanced the growth of
sympathetic neurites *in vitro*, as assessed by standardized quantification. Our observations of a concentration-dependent increase in neurite number and percentage of cells growing neurites demonstrate that IL-17 can significantly affect neurite architecture. Although several cytokines have been demonstrated to exert independent neurotrophic effects (Kannan *et al.*, 1996; Temporin *et al.*, 2008; de Araujo *et al.*, 2009), this is the first study, to our knowledge, which identifies IL-17 as a neurotrophic factor.

Pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-17 are released in high levels surrounding areas of tissue damage. IL-17 plays a pivotal role in the pathology of IBD; tissue biopsies from IBD patients have elevated levels of Th17 cells and IL-17 (Fujino *et al.*, 2003) and genetic mutations in the Th17 axis confer protection from colitis (Duerr *et al.*, 2006). It would follow from the *in vitro* data in this study that areas of active inflammation, expressing high levels of IL-17, are invaded by sympathetic fibres. Several studies have demonstrated an increased density of tyrosine-hydroxylase immunopositive fibres in animal models of IBD, in addition to Crohn’s and colitis patients (Birch *et al.*, 2008; Bai *et al.*, 2009). It is possible that local increases in IL-17 levels at the mucosa and lamina propria would cause a proliferation of sympathetic fibres *in vivo*.

Certain cytokines (IL-4, IL-6 and IFN-γ) have been shown to enhance the growth of neurites initiated by endogenous and recombinant neurotrophins (Golz *et al.*, 2006). NGF was present at 50 ng/mL in all experimental cultures, and IL-17 without NGF was insufficient to support neuronal survival and neurite outgrowth (data not shown). Therefore, it is possible that IL-17 is acting synergistically by enhancing the actions of
the NGF in the bath. On the other hand, since inhibition of NF-κB reversed the effect of IL-17 and NGF does not act via this pathway, it seems plausible that IL-17 is enhancing neurite outgrowth via a novel mechanism, independent of NGF activity.

Other Th17-driven diseases, particularly rheumatoid arthritis (RA), are characterized by increases in tyrosine hydroxylase immunoreactivity not only into the actively inflamed synovial capsules, but into distant lymphoid structures such as the spleen (Pernis, 2009; Lorton et al., 2009). Nerve fibre density is greater in skin lesions of patients with psoriasis, a disease known to involve recruitment of T cells to the site of injury and increased levels of IL-17 (Cevikbas et al., 2007). It seems that both elevated circulating levels of cytokines and localized concentrations of immune cells can induce the ingrowth of sympathetic nerves. The fact that this phenomenon is observed in diseases known to depend on IL-17 lends strength to the theory that this cytokine has important effects on neuronal structure.

4.3 Ca\(^{2+}\) currents and IL-17-induced outgrowth

In order to investigate a potential mechanism for the observed effect, the effect of IL-17 on inward Ca\(^{2+}\) currents was studied using patch clamp electrophysiology, Ca\(^{2+}\)-imaging, and pharmacological techniques. It has previously been established that a decrease in the frequency or amplitude of inward Ca\(^{2+}\) current can enhance the growth of Xenopus spinal axons (Gomez et al., 2001) and cerebellar Purkinje neurites (Schilling et al., 1991). An increase in intracellular Ca\(^{2+}\) underlies the arrest of neurite outgrowth when locus celuleus neurons encounter a stop signal (Moorman & Hume, 1993).
Overnight incubation with IL-17 significantly attenuated inward current through VGCCs, as assessed by voltage clamp electrophysiology; results which were mirrored using fluorometric Ca\textsuperscript{2+} imaging. Not only did these effects occur at the level of the cell body, but, after two-day incubation in IL-17, Ca\textsuperscript{2+} imaging within the growth cones directly revealed that the amplitude of inward Ca\textsuperscript{2+} flow upon depolarization with high K\textsuperscript{+} solution was significantly reduced.

This finding establishes a potential mechanistic explanation for IL-17-induced increased outgrowth, and also confirms our ability to correlate changes seen in neuronal cell bodies to more distal locations. Conflicting studies regarding the increase or decrease in neurite growth as correlated with growth cone [Ca\textsuperscript{2+}] have led to the hypothesis that a certain “set point” of Ca\textsuperscript{2+} is required for optimal neurite elongation (Bolsover, 2005). It may be that the reduced Ca\textsuperscript{2+} currents induced by IL-17 brings the overall “set point” closer to the optimal level for growth in these SMG neurons. Alternatively, specific alterations in N-type VGCC function – such as physical interactions with extracellular matrix proteins – may be responsible for the effect (Sann et al., 2008).

4.4 Voltage-gated Ca\textsuperscript{2+} channels and outgrowth

Previous studies in our lab have demonstrated that N-type VGCCs are the principal isoform present in SMG neurons. Given this fact, and the fact that N-type VGCCs have an established role in the transduction of neurite outgrowth signals (Heng et al., 1999; Ryan et al., 2007; Sann et al., 2008), the specific blocker ω-conotoxin GVIA
was added to the culture medium to determine if a blockade of inward $I_{\text{Ca}}$ would mimic the actions of IL-17. Indeed, a sub-maximal concentration of CTX caused an increase in neurite outgrowth similar to that evoked by IL-17. 300 nM CTX is sufficient to totally inhibit N-type current in SMG neurons (Motagally et al., 2009a), therefore 30 nM was chosen to provide a partial blockade. Incubation with the N-type blocker also enhanced outgrowth in systems where an arrest in outgrowth depended on inward flow of $\text{Ca}^{2+}$ — whether activated by depolarization (Ryan et al., 2007) or a laminin-mediated stop signal (Sann et al., 2008). The present study points to a similar effect of VGCC blockade.

The possibility that steric interactions of CTX with the N-type VGCCs were responsible for altering neurite outgrowth (Sann et al., 2008), potentially via a mechanosensitive function of this channel (Calabrese et al., 2002), led us to study the role of depolarization-induced $\text{Ca}^{2+}$ influx itself. Tetrodotoxin, which blocks voltage-dependent Na$^+$ channels and therefore depolarization, was added to the culture medium to prevent the activity of N-type VGCCs without directly interfering with the VGCC subunits. Like culturing with CTX, TTX treatment enhanced the growth of neurites in a parallel manner to that of IL-17. These results confirm that the voltage-dependent influx of $\text{Ca}^{2+}$ underlies the brake on neurite outgrowth, and argue against the idea that physical interactions of N-type VGCCs are responsible for the effect.

4.5 IL-17 acts through the NF-κB pathway

NF-κB inhibition reversed both the electrophysiological and morphological changes caused by IL-17. Addition of the IκK inhibitor SC-514 prevented the inhibition
of inward $I_{\text{Ca}}$ as assessed by voltage clamp electrophysiology and $\text{Ca}^{2+}$ imaging, as well as the increase in neurite growth parameters induced by IL-17. The finding that inhibition of the NF-κB pathway reverses the observed outgrowth is an intriguing one, and seems to indicate that activation of NF-κB signaling is a necessary step in IL-17-induced neurite sprouting. NF-κB is an effector molecule common to many pro-inflammatory stimuli (Sarkar et al., 2008; Wong & Tergaonkar, 2009), and has been demonstrated to be a downstream effector of IL-17 signaling (Kehlen et al., 2001).

Several neuronal processes are under the control of the NF-κB pathway, including consolidation of fear responses (Lubin & Sweatt, 2007), spatial memory (Barger et al., 2005), and expression of neurotransmitter receptors (Heckscher et al., 2007). Recently, the role of NF-κB activation in enhancing neurite outgrowth has been a subject of interest. Blocking the NF-κB pathway drastically inhibits the growth of neurites from nodose ganglion neurons (Gallagher et al., 2007), hippocampal neurons (Sole et al., 2004) and PC12 cells, a culture model of sympathetic neurons (Gutierrez et al., 2005). Previous research has implicated NF-κB in the TNF-α-induced inhibition of VGCCs in SMG neurons (Motagally et al., 2009a). Our results show a similar involvement of NF-κB in $I_{\text{Ca}}$ inhibition, as well as a novel effect on outgrowth of sympathetic neurites.

These data provide a compelling argument for mechanistic commonality between the two effects of IL-17. Although IL-17 may both inhibit inward $\text{Ca}^{2+}$ current and enhances neurite growth through separate pathways downstream of NF-κB activation, the most parsimonious explanation for these results is that the phenomena are...
interrelated. We propose a model wherein NF-κB-dependent inhibition of N-type VGCC function relieves a constitutive, Ca\(^{2+}\)-mediated stop signal and results in increased growth and elongation of sympathetic neurites.

4.6 Involvement of glial cells

It could be argued that indirect effects of IL-17 on the glial cells present in dissociated SMG cultures are responsible for the enhanced outgrowth. However, several lines of evidence dispute this. Using both a three-day and a seven-day assay of outgrowth, the depletion of the cultures of their glia did not significantly alter the effect of IL-17. Despite a 65% reduction in glial numbers in the short-term culture model and an 89% reduction in the long-term model, the relative increase in outgrowth caused by IL-17 treatment was unaffected. It must be conceded that it is not possible in our culture system to eliminate 100% of the glial cell population, and thus the contribution of these cells cannot be fully dismissed.

In further support for a direct action of IL-17 (rather than an indirect action through glial cells) are the observations of directional growth towards an IL-17-impregnated bead. The increased growth of neurites towards a focal source of IL-17 demonstrates that not only does IL-17 cause a generalized increase in growth, but it also exerts a chemotactic effect on sympathetic neurites. If the mechanism of action of IL-17 were activation of glial cells to produce increased levels of canonical growth factors, it would be difficult to explain a specific chemoattractive effect on individual nerve fibres. Directional growth of neurites towards a target relies on attractive signals acting at the
level of the growth cone; diffusible factors and substrate proteins interact locally with the neurite, resulting in an increased rate of growth along the concentration gradient of the attractant. The present results fit the model that direct activation of cytokine receptors expressed on the surface of neurons can result in outgrowth independently of glial cells.

4.7 Broader significance: the SNS and IBD

The involvement of the SNS in regulating GI inflammation and immune system activation is well-established (Straub et al., 2008; Vasina et al., 2008; Boissé et al., 2009). Balance seems to be key to normal physiological function; over- or under-activation of the SNS leads to pathology of the GI tract, particularly in states of inflammation (Green et al., 1998; Maule et al., 2007; Elenkov, 2008). The levels of pro-inflammatory cytokines such as IL-17 in areas of active inflammation and systemically may act to upset the delicate balance of SNS activation. An IL-17-induced hyperinnervation of the gut could potentially be responsible for over-activation of the SNS. During chronic inflammatory disease, a so-called “β- to α-adrenergic shift” has been found to occur; shifting from anti-inflammatory β-adrenoceptors as the targets of noradrenaline to the pro-inflammatory α-receptors (Straub et al., 2006). A proliferation of sympathetic fibres together with a preponderance of pro-inflammatory downstream effectors could have a role in maintaining a hyperactive immune state in IBD.

Several studies have demonstrated a deficit in sympathetic activity (reduced NA release; Blandizzi et al., 1993; Jacobson et al., 1995; Swain et al., 1991), as well as effector function (defective vasoconstriction (Neshat et al., 2009)) during IBD, which
may contribute significantly to the observed pathologies. For example, ineffective sympathetic vascular regulation would result in a lack of mucosal perfusion, precipitating a breakdown in barrier function. As discussed earlier, elevated levels of IL-17 would seem to induce the proliferation of new sympathetic fibres in the gut. The question would then remain as to whether the new fibres had optimal function. Studies in DSS colitis (Motagally et al., 2009b), as well as human CD patients (Magro et al., 2002) have shown reduced NA release, which seems to indicate a decrease in the number of sympathetic nerve fibres.

A paradoxical effect whereby IL-17 causes an increase in nerve fibre density yet a decrease in neurotransmitter release is an intriguing possibility. Our findings indicate that IL-17 incubation not only enhances outgrowth, but also reduces inward I_{Ca}. The importance of inward current through VGCCs in neurotransmitter release (and thus effective function) of sympathetic neurons is well-documented (Smith & Augustine, 1988; Lledo, 1997; Jackson & Cunnane, 2001). In the GI tract, release of neurotransmitter from sympathetic varicosities depends on N-type VGCCs (Smith & Cunnane, 1997). It could be that despite an increased ingrowth of sympathetic fibres, the new neurites are functionally compromised. Ongoing inhibition of VGCC function would result in reduced neurotransmitter release; even if there is an increased neurite proliferation in the gut wall, nerve function and therefore SNS immunomodulation and control of GI function may be defective. Furthermore, once inflammation subsides, an increased number of sympathetic nerve terminals in the gut wall – no longer subject to inhibition of I_{Ca} – could play a role in ongoing GI dysfunction.
4.8 Future directions

Further studies will focus on the precise downstream effectors of the NF-κB pathway that are responsible for enhancing outgrowth. Recently, it has been shown that the phospho-Serine 536-p65 subunit of NF-κB enhances neurite growth while unphosphorylated p65 does not (Gutierrez et al., 2008). In the future, Western blotting and pharmacological studies will be used to investigate the involvement of p65 phosphorylation in the effects of IL-17.

Future research will also focus on correlating the in vitro changes evoked by cytokine treatment to the in vivo ingrowth of sympathetic neurites in damaged areas of the intestine, and the functional significance of the alterations in neurite architecture. A possible role for tonic sympathetic activity in visceral hypersensitivity could be a rich avenue for further study, and the role of the alterations in sympathetic neurite structure in dampening or enhancing the ongoing inflammatory state in the intestine during IBD will be interesting to explore.
Chapter 5

Reference List


