A STUDY OF MECHANISMS UNDERLYING INFLAMMATION-INDUCED
ABNORMAL NOCICEPTIVE SIGNALING FROM THE COLON IN MODELS OF
IRRITABLE BOWEL SYNDROME (IBS) AND INFLAMMATORY
BOWEL DISEASE (IBD)

by

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A thesis submitted to the Department of Physiology in conformity with the
requirements for the degree of Doctor of Philosophy

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ABSTRACT

Abdominal pain is a common symptom of inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). Although the extent differs, visceral inflammation is thought to play a significant role in nociception in these disorders. This thesis describes studies examining mechanisms of inflammation-induced changes in nociceptive signaling from the colon using human colonic biopsy and animal models of the conditions.

*Citrobacter rodentium* infection in mice produced profound colitis at day 10 post-infection which resolved by day 30. Perforated current clamp recordings showed that inflammation induced hyperexcitability of colonic DRG neurons that persisted at day 30 post-infection. Similarly, multi-unit afferent nerve recordings revealed enhanced firing of colonic afferents following colon distension at this time. In voltage clamp studies, suppression of $I_A$ $K^+$ currents in post-infected *C. rodentium* neurons was observed. Combining water-avoidance stress (WAS) and *C. rodentium* infection exaggerated these effects. Interactions between proteases and stress mediators underlie these actions. *In vivo* studies revealed WAS combined with *C. rodentium* post-infection induced visceral hyperalgesia and allodynia.

A separate series of studies examined the possible role of cysteine proteases in post-infectious IBS. The cysteine protease cathepsin-s (Cat-S) induced neuronal excitability and, provoked visceral hypersensitivity in mice. Human IBS supernatants increased neuronal excitability, but this was reversed in
neurons pre-treated with the cysteine protease inhibitor E-64. Together these data suggest that Cat-S is a secreted neuromodulator in human IBS supernatants and could be important in nociceptive signaling in IBS.

In studies examining whether similar mechanisms operate in a traditional inflammatory condition, IBD, human ulcerative colitis (UC) supernatants showed elevated TNF-α levels. Exogenous TNF-α and UC-supernatants increased colonic nociceptor excitability, which was attenuated in neurons from TNFR knock-out animals. TNF-α and UC-supernatants both increased TTX-R Na_v1.8 and suppressed I_A and I_K K⁺ currents.

Together these results suggest that inflammation significantly increases and sustains peripheral nociceptive signaling in IBD and IBS. These effects involve changes in the properties of nociceptive DRG neurons through actions of specific secreted factors which modulate specific voltage-gated ion channels. Chronic stress exaggerates these changes through synergistic actions of stress hormones and local mediators, suggesting an interplay between central and peripheral mechanisms.
CO-AUTHORSHIP

The majority of the work and data described in this thesis was designed and performed by me, Charles O. Ibeakanma. The isolation and culture of *Citrobacter rodentium* strains used for induction of colitis in chapters 2 and 3 were performed by Dr. Nancy Martin and colleagues in her lab. Histopathology data presented in chapters 2 and 3 were collected by Dr. David Hurlbut, while protease activity data presented in chapter 3 were measured by Dr. Nigel Bunnett and his colleagues at the University of California and Dr. Nathalie Vergnolle at INSERM Unité 563 Centre de Physiopathologie de Toulouse Purpan, Toulouse, France. Laser-capture microdissection of Fast-Blue labeled colonic DRG neurons and quantitative RT-PCR data presented in chapter 3 were performed by Todd McDonald. Dr. Fernando Ochoa-Cortes contributed a portion of the electrophysiological data presented in chapter 3, while Drs. Ian Spreadbury and Nicolas Cenac performed the visceromotor response (VMR) to colorectal distension (CRD) experiments. Dr. Marcela Miranda-Morales collected afferent nerve recordings data presented in chapters 2 and 3. The writing of the initial draft of manuscripts for chapters 3, 4 and 5 was done solely by me. In addition, I contributed to the writing of those of chapter 2 together with Dr. Stephen Vanner who provided editorial assistance for all the chapters.
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I would also like to use this opportunity to thank my thesis committee members – Drs. Michael J. Beyak, Chris Ward and Nancy Martin. This thesis would not have been completed without your invaluable input.

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Being able to maintain sanity all these years and to remain optimistic in pursuing my life set goals is as a result of the loving friendship I received and continue to receive from my family. To my wife, Sipho Ibeakanma, I say there is no other like you. You are everything a woman is to a man. I am incredibly blessed to have you as a life partner. You are always there to provide comfort and hope. Through some special gift of yours, you always manage to put smiles on my face, especially on those tough days when I had returned from the lab frustrated. I love you now and will always do. To my son, prince Chinedum Justin (CJ) Ibeakanma, you mean the world to me. I cannot forget your timings for “attention seeking”. You always choose to show me the new tricks you learnt at the Daycare or insist I watch “Tree house” or even play Basket ball with you, at those times that I would rather be sitting at the reading table. How could Daddy be studying instead of playing with me, I guess you would be thinking. You know what CJ? Those were the most fun moments of my life. Thank you for bringing me joy. You are my best friend and I love you. During the course of writing this thesis, I had the honor of my mother (the Queen mother) visiting from Nigeria. Mama, thank you for the visit and all your support.

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<td>acid-sensitive ion channel</td>
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<td>α-GCR</td>
<td>alpha-glucocorticoid receptor</td>
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<td>APC</td>
<td>antigen presenting cells</td>
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<td>4-AP</td>
<td>4-aminopyridine</td>
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<td>5-ASA</td>
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<td>β2-AR</td>
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<td>CRD</td>
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<td>complementary deoxyribonucleic acid</td>
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<td>ELISA</td>
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<td>FB</td>
<td>fast-blue</td>
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<td>fetal calf serum</td>
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<td>HPA-SA</td>
<td>hypothalamic pituitary adrenal-sympathoadrenal axis</td>
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<td>IECs</td>
<td>intestinal epithelial cells</td>
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<td>INF-γ</td>
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<td>I.P.</td>
<td>intra peritoneal</td>
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<td>transient inactivating A-type current</td>
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<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
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<td>IBS</td>
<td>irritable bowel syndrome</td>
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<td>$I_K$</td>
<td>sustained non-inactivating K-type current</td>
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<td>$k$</td>
<td>slope factor</td>
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<td>voltage-gated potassium channel</td>
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<tr>
<td>kHz</td>
<td>kilohertz</td>
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<td>MAPK</td>
<td>extracellular signal-regulated kinase</td>
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<td>myeloperoxidase</td>
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<td>prostaglandin $E_2$</td>
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<td>PI-IBS</td>
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<td>reverse transcriptase polymerase chain reaction</td>
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<td>second</td>
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<td>transient receptor potential</td>
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CHAPTER 1: GENERAL INTRODUCTION
Multi-cellular organisms have a system of organs in the gastrointestinal (GI) tract that enables them to ingest, digest, and absorb nutrients from food, and eventually eliminate unwanted materials (Knowles & Aziz, 2009). These essential functions, which for the most part occur involuntarily, are facilitated by the presence of a well organized nervous system. The GI tract also has the capacity to provide conscious awareness (sensation of pain) that alerts an organism of impending tissue damage which could hamper these vital physiological functions. Normally, it involves interplay between immune, peripheral and central nervous systems (Knowles & Aziz, 2009). However, when such important and protective signaling events become disrupted by a pathological process, especially over a long period of time, the organism can undergo a measure of incapacitation due to the resultant discomfort and pain. This altered sensory signaling is a common feature of disorders of the GI tract, including inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) (Cain et al., 2006). Tissue inflammation of varying degree is present in the human and animal models of these conditions and is thought to play a role in the sensory alterations. This thesis examined mechanisms by which visceral inflammation contributes to alterations in nociceptive signaling from the colon in human and animal models of IBD and IBS.
AFFERENT INNERVATION OF THE VISCERA

The GI tract has a rich supply of both intrinsic and extrinsic sensory nerves. The intrinsic nerves are comprised of a network of plexuses, the myenteric and submucosal plexuses, which together form the enteric nervous system (ENS). The ENS is found within the smooth muscle layers of the gut (myenteric plexus) and in the submucosa (submucosal plexus). The extrinsic nervous system links the viscera to the CNS through the vagal and spinal nerves (Grundy et al., 2006). These afferent nerves travel in bundles of mixed motor and sensory fibers, and follow the paths of parasympathetic and sympathetic outflows from the brain to innervate the GI tract. Through the sensory fibers, they relay information relating to both painful and non-painful sensations.

ANATOMICAL ORGANIZATION OF VISCERAL EXTRINSIC (AFFERENT) NERVES

The entire viscera receive innervation from the extrinsic nervous system, which is composed of two sets of primary afferent nerves - vagal and spinal afferents (via the splanchnic and pelvic nerves) (Figure 1-1). Vagal afferent fibers are concentrated mainly in the upper GI regions, with the innervation density decreasing towards the lower regions (up to the ascending and transverse colon where it terminates). On the other hand, the splanchnic nerves supply the entire viscera, while pelvic nerve innervations are concentrated in the lower GI regions (descending colon and rectum) only (Sengupta, 2009).
Splanchnic and pelvic spinal afferent fibers follow the paths of sympathetic and parasympathetic efferent outflows from the brain respectively, to innervate the gut. Afferent terminals of both fibers are localized throughout the viscera, with their cell bodies residing in the dorsal root ganglia of the thoracic and lumbar (between levels C1 to S4) segments of the spinal cord. These neurons are pseudo-unipolar in nature, i.e. they give rise to single axons that divide into central and peripherally projecting branches. The central branch courses through prevertebral ganglia, to synapse mainly in the superficial lamina I and deeper laminae V – VII of the dorsal horn (Grundy et al., 2006). From there, second order axons travel in either the spinothalamic, spinoreticular, spinohypothalamic or spinomesencephalic tracts to different areas of the brain (Almeida et al., 2004). The peripheral branch travels through the paravertebral ganglia to innervate the different organs of the GI tract. Each organ receives afferent fiber innervations from a number of DRGs in different spinal segments that also supply other organs, including the skin, to the extent that considerable overlap exists (Berthoud et al., 2004; Knowles & Aziz, 2009).

CLASSIFICATION OF VISCERAL AFFERENT NEURONS

An important feature of visceral afferent neurons is that their properties are very heterogeneous. They have been classified based on a number of different criteria, including anatomical localization of their peripheral terminals in the gut,
Figure 1-1 Schematic representation of extrinsic innervations of the GI tract. The GI tract is innervated by the extrinsic nervous system composed of two sets of primary afferent nerves - vagal and spinal afferents (splanchnic and pelvic nerves). Both vagal and spinal afferents supply the thoracic and upper abdominal regions; the small intestine, the ascending and transverse colon receive innervations from the vagus and splanchnic nerves; while the descending colon and rectum receive innervations from the splanchnic and pelvic nerves. Splanchnic fibers travel in the sympathetic trunk while the pelvic fibers run in the parasympathetic trunk. Adapted from Beyak et al., 2006.
their mechanosensitivity responsiveness and morphology (size) of the cell body / axons as summarized below.

**Classification based on terminal localizations:** Peripheral terminals of afferent fibers are located in all layers of the visceral wall where they are positioned to respond to local chemical and mechanical stimuli (Gebhart, 2000) and as such, are grouped into four categories: mucosal, muscle, muscle/mucosal, and serosal/mesenteric afferents (Figure 1-2) (Brierley et al., 2004; Sengupta, 2009). The anatomical localization of these terminals was made possible by the use of electrophysiological techniques and tracer dyes that fill fine branches of the nerve trunk (Berthoud et al., 2004; Tassicker et al., 1999). For example, Tassicker and colleagues used biotinamide to visualize peripheral branches of mesenteric nerve trunk that supply guinea-pig small intestine (Tassicker et al., 1999). When applied to *in vitro* solutions, the dyes were rapidly taken up by the nerve trunk and conveyed retrogradely to their terminals in the walls of the viscera. Tracers such as Dil, neurobiotin, wheatgerm agglutinin-conjugated horseradish peroxidase or dextran have also been employed by a number of other investigators (Berthoud et al., 2004). In electrophysiological studies, mucosal responses to mechanical stimuli (e.g. light stroking) or chemical agents were employed to demonstrate the existence of afferent fibers in the mucosal and submucosal layers of the colon (Brierley et al., 2004; Lynn & Blackshaw, 1999).
Figure 1-2 Localization of peripheral afferent terminals in layers of GI wall. Cartoon illustrating presence of peripheral afferent terminals in all layers of the viscera where they are positioned to respond to local chemical and mechanical stimuli. Numbers 1 – 4 represent the different groups of visceral afferent endings based on their localization. Adapted from Knowles & Aziz, 2009.
Reports indicate that the majority of spinal afferent endings in the viscera are free and unmyelinated (Blackshaw & Gebhart, 2002), with a few specialized structures found in pelvic spinal afferents. A large percentage of spinal afferents are mechanosensitive, responding to luminal distension or stretching of the gut (Sengupta, 2009); whereas others, constituting a smaller percentage, are chemosensitive in nature, responding to stimuli such as pH, nutrients, inflammatory mediators etc., (Berthoud et al., 2001; Brierley et al., 2005; Lynn & Blackshaw, 1999).

**Classification based on mechanosensitivity:** Spinal visceral afferents can also be classified based on their mechanosensitive responsiveness as:

1) **Tonic (or wide dynamic range) mechanoreceptors** display spontaneous activity at rest. The spontaneous discharges at rest are attributed to tonic activation of 5-HT3 and TRPV1 receptors, because specific antagonists of these receptors attenuated the activity in *in vitro* recordings (Hillsley et al., 1998; Rong et al., 2004); Tonic mechanoreceptors respond linearly to tensile muscle distension beginning from low thresholds. In addition, they can sense the fullness of hollow organs such as the stomach, colon and rectum, and are thought to play a part in discomfort and pain (for example, during inflammation) since they can be activated well into the noxious range (Blackshaw & Gebhart, 2002; Knowles & Aziz, 2009; Sengupta & Gebhart, 1994).

2) **Phasic (or high threshold) mechanoreceptors** have low resting activity. They are considered mechanoreceptors because they respond to mechanical
stimuli (e.g. organ distension), but only in the noxious range (Knowles & Aziz, 2009; Sengupta et al., 1990). Their terminal endings have reportedly been found at sites of intense mechanosensitivity, such as in the mesentery and serosa (Tassicker et al., 1999). In addition, they respond directly to a number of inflammatory mediators such as capsaicin, bradykinin, prostaglandins, leukotrienes, histamine, free radicals etc., and are therefore also considered to be chemosensitive (Blackshaw & Gebhart, 2002; Knowles & Aziz, 2009). The wide dynamic range and high threshold fibers are thought to be primarily involved in visceral nociceptive signaling. Evidence supporting this notion was provided by studies that combined tracer and electrophysiological techniques (Ozaki & Gebhart, 2001; Sengupta et al., 1990).

3) Silent nociceptors (or mechanically insensitive afferents) – these afferents are silent at rest, and only become mechanosensitive after long periods of noxious stimulation, e.g., during inflammation (Dmitrieva & McMahon, 1996). They are found in almost all visceral tissues. In somatic tissues, they are thought to serve as chemonociceptors (Blackshaw & Gebhart, 2002); but no one knows if they respond similarly in the viscera.

**Classification based on size:** As a result of differences in their functional and morphologic characteristics, the cell bodies (neurons) of DRGs are classified into large, medium and small sized neurons (Almeida et al., 2004). These correspondingly give rise to the myelinated (Aβ), thinly myelinated (Aδ) and unmyelinated (C) afferent fibers that innervate the gut. Aβ fibers have the largest
diameter (> 10 µm). They conduct sensory information rapidly (velocity: ~ 30 – 100 m/s) and are mainly involved in the detection of innocuous stimuli. Thus, they do not participate in visceral pain signaling (Djouhri et al., 1998; Julius & Basbaum, 2001). Aδ fibers are of medium size (2 – 6 µm), with an intermediate conduction velocity of 12 – 30 m/s; while C fibers are small in size (0.4 – 1.2 µm) and conduct sensory information at a slow velocity of 0.5 – 2 m/s. Aδ and C fibers are thought to be directly involved in mediation of pain evoked by noxious stimuli (Almeida et al., 2004). The C-fibers in particular are known to respond to intense and noxious stimuli, and are therefore referred to as nociceptors (Lee et al., 2005). A prominent feature of nociceptors is that they exhibit a high threshold for activation, and are in most cases quiescent until activated by noxious stimuli (Julius & Basbaum, 2001; Lee et al., 2005). Furthermore, nociceptors are polymodal in nature, responding to more than one noxious stimulus (mechanical, chemical etc.) (Gebhart, 2000; Julius & Basbaum, 2001; Lee et al., 2005).

In the patch clamp studies described in this thesis, we classified DRG cell bodies according to size, and only conducted experiments on small neurons that give rise to C-fibers innervating the inflamed colon (i.e. small DRG neurons [<40 pF; pico-Farad]) because these have been shown to exhibit properties consistent with nociceptors (Beyak & Vanner, 2005; Moore et al., 2002; Yoshimura & de Groat, 1999). Chief amongst these properties are 1) they (nociceptors) are activated by the algogenic compound capsaicin, 2) they display tetrodotoxin-resistant (TTX-R) action potentials and, 3) during membrane depolarization, they
display a phasic pattern of firing (i.e. they fire a single action potential) at threshold value of current sufficient to elicit the first action potential (i.e. at the rheobase).

RECEPTORS FOR INFLAMMATORY MEDIATORS EXPRESSED ON AFFERENT NEURONS OF THE GUT

Many different kinds of membrane receptors are expressed on visceral afferents and their cell bodies, including G-protein coupled receptors, ligand-gated ion channels and growth factor receptors. Expression of these receptors was confirmed using electrophysiological, cellular and molecular biological techniques (Grundy, 2004; Ward et al., 2003). The receptors are capable of interacting with a wide variety of endogenously or exogenously generated substances, such as 5-HT, PGE2 and tryptase, to effect cellular functions, including nociceptive signaling in the gut (Grundy, 2004). Several of the mediators come from a variety of cell types such as lymphocytes, platelets, macrophages, mast cells, glia, fibroblasts, muscle cells, neurons etc., activated during conditions of inflammation or injury (Mosmann & Coffman, 1989). Agonists coupling to the receptors may cause activation of the afferent neurons through gating of expressed ion channels (e.g. ligand-gated / mechanosensitive ion channels); or activation through stimulation of intracellular signaling events capable of inducing increased synthesis / insertion of new channels into the cell membrane, transcription and/or release of other mediators capable of modulating
existing membrane channels, or modulating the existing membrane bound
cannels through phosphorylation, dephosphorylation etc., or other cellular
processes (Grundy, 2004). The end result of these processes is increased
nociceptive signaling through enhanced responsiveness of the afferents to
mechanical and chemical stimuli.

INITIATION, MAINTENANCE AND TRANSMISSION OF NOCICEPTIVE
SIGNALING IN AFFERENT NEURONS OF THE GUT

Action potentials are the principal signals elicited in afferent neurons in
response to axon terminal stimulation by inflammatory mediators or other
environmental stimuli. Following stimulation, one of two important events
(sometimes both) that influence the electrical activity of afferent neurons occurs
at the terminal endings (Figure1-3):

1) Mediator coupling to specific receptors may trigger intracellular
events that lead to the activation and conversion of the signals (transduction) into
a "generator potential" by ionotropic receptors / mechanosensitive channels (for
example, TRP channels and ASIC). This produces a slight membrane
depolarization due to the influx of cations into the cell (Figure 1-3A; 1). The
depolarization in turn induces opening of voltage gated ion channels like Na+
channels, allowing more ions (Na+) into the cells and thus depolarizing the
membrane further. When the magnitude of membrane depolarization is sufficient
Figure 1-3 Schematic illustration of events leading to increased afferent neurons excitability. (A) Inflammatory mediators activate intracellular pathways through coupling to their specific receptors, leading to either sensitization and/or activation of ionotropic channels (e.g. TRPV1) to elicit generator potentials and the subsequent action potential eletrogenesis (1), or intracellular pathways that alter the biophysical properties of voltage gated channels (e.g. Na+ and K+) to increase nociceptive signaling through changes in the frequency and shape of action potential discharges (2). (B) Representative trace of action potential showing the different characteristic parameters (amplitude, width and activation threshold) that could change as a result of modulation of voltage-gated ion channels by inflammatory mediators to reflect changes in neuronal excitability.
to reach a threshold for activation, action potentials are generated. The generated action potentials are then rapidly propagated along the axons for central processing of encoded information (Figure 1-3A). The initiated intracellular signal could also induce alteration in the structural configuration and property of the ionotropic receptors leading to their sensitization (Amadesi & Bunnett, 2004). Because these receptors can also respond to mechanical stimuli, coincident activation by stimuli such as mucosal distension/stretching causes greater influx of ions that lead to an overall enhancement in the afferent neurons activity and hypersensitivity (Cenac et al., 2007).

2) Coupling of mediators to their receptors (e.g. G-protein coupled receptors [GPCRs]) could also activate intracellular pathways that lead to the modulation of membrane bound voltage gated ion (\(\text{Na}^+\) and \(\text{K}^+\)) channels (Figure 1-3A; 2) and/or other channel types such as TRP and \(\text{K}_2\text{P}\). This process may occur through phosphorylation, dephosphorylation etc., or other cellular processes, that eventually induce changes in the biophysical properties of the channels and as a result, changes in their gating activities. The altered gating activity reflects increased or decreased frequency of action potential discharges, and alterations in the shape and characteristic of the action potentials generated (i.e. changes in the width, amplitude, activation threshold etc.) (Figure 1-3B). These events ultimately give rise to increased neuronal excitability and hence increased nociceptive signaling. For example, \textit{in vitro} activation of protease activated receptor 2 (PAR2) expressed on colonic nociceptive DRG neurons lead
to hyperexcitability of the neurons (Kayssi et al., 2007). In these studies, suppression of delayed rectifier \( I_k \) currents through PKC and ERK dependent pathways were implicated.

ION CHANNELS MEDIATING NOCICEPTIVE SIGNALING (EXCITABILITY) IN VISCERAL AFFERENT NEURONS

The roles of sodium and potassium conductances in the generation, propagation and termination of action potentials, as well as, the regulation of membrane excitability have long been established (Hodgkin & Huxley, 1952).

**Voltage-gated sodium (Na\(_\nu\)) channels:** Opening of Na\(_\nu\) channels by depolarizing stimuli allows entry of Na\(^+\) ions into the cells and this has been shown to mediate the rapid upstroke of the action potential (Matsutomi et al., 2006). The timely voltage-dependent inactivation of the Na\(^+\) channels and the simultaneous activation of K\(^+\) channels are responsible for termination of the action potentials. Multiple types of Na\(_\nu\)\(^+\) channel \( \alpha \)-subunits are expressed in visceral sensory neurons, and based on their selective sensitivity to the natural puffer fish toxin, tetrodotoxin (TTX), are grouped into TTX-sensitive (TTX-S) and TTX resistant (TTX-R) channels (Matsutomi et al., 2006). While TTX-S channels (Na\(_\nu\)1.1, Na\(_\nu\)1.2, Na\(_\nu\)1.3, Na\(_\nu\)1.6 and Na\(_\nu\)1.7) are broadly expressed on all types of spinal afferent neurons, TTX-R channels (Na\(_\nu\)1.8 and Na\(_\nu\)1.9) are preferentially localized on the pain sensing afferent neurons (Benn et al., 2001). In these neurons, currents mediated by TTX-R Na\(_\nu\)1.8 channels have particularly been
suggested to be responsible for action potential initiation. They have also been implicated in repetitive firing due to their rapid re-priming behavior (Akopian et al., 1999); whereas Na\textsubscript{v}1.9 currents are thought to be involved in setting membrane potential and threshold for action potential generation. Inflammatory mediators are thought to particularly modulate TTX-R Na\textsubscript{v}1.8 currents. For example, application of PGE2 to selected colonic DRG neurons increased the currents (Gold et al., 2002) through activation of cyclic AMP-protein kinase A (cAMP/PKA) signaling pathways (England et al., 1996). Similarly, it was recently shown that TNF-\textgreek{a} modulates TTX-R Na\textsuperscript{+} currents to increase neuronal excitability in mouse DRG neurons through activation of p38 MAPK signaling pathway (Jin & Gereau, 2006). When considered together, these results support a role for sodium conductance in the modulation of neuronal excitability.

**Voltage-gated potassium (\textit{K}\textsubscript{v}) channels:** As stated earlier, the time and voltage dependent opening of these channels, in part, play a critical role in terminating action potentials, and thus are important in determining the excitability of afferent neurons. They permit the cell membranes to accommodate to depolarizing stimuli. In other words, they help to re-polarize the membrane, and thus hinder frequent action potential firing (Beyak, 2010). Based on their inactivation characteristics, currents mediated by these channels in response to depolarizing stimuli are broadly classified into a rapidly inactivating (I\textsubscript{A}) and very minimally or in most cases non-inactivating (I\textsubscript{K} or delayed rectifier) currents. I\textsubscript{A} currents have hyperpolarized thresholds for inactivation, meaning that few
channels are available for opening at or near the resting membrane potential. Studies using specific blockers have demonstrated their importance in modulating membrane excitability in visceral afferent neurons. For example, blockage of $I_A$ currents with specific antagonist 4-aminopyridine induced a significant increase in action potential discharges in response to depolarizing pulses (Moore et al., 2002; Stewart et al., 2003). $I_K$ currents inactivates very minimally over time. They have a more depolarized threshold for activation and are thought to play a role in setting the resting membrane potential. Suppression of $I_K$ currents depolarizes the membrane and increases the width of action potentials (Yoshida et al., 2007).

Other voltage-activated currents that may play a part in determining the excitability of visceral afferents include those mediated by calcium-activated potassium channels and the hyperpolarization-activated cyclic nucleotide-gated channels ($I_H$ currents) (Cordoba-Rodriguez et al., 1999; Robinson & Siegelbaum, 2003). It is possible that these channels play a role in generating afterhyperpolarization and may respond to numerous inflammatory mediators. However, to date, direct evidence for their presence in visceral afferents is lacking.

**Two-pore domain $K^+$ channels ($K_{2P}$):** $K_{2P}$ channels are normally active within physiological ranges of membrane potential and have been shown to be the molecular source of leak (background) $K^+$ currents (Goldstein et al., 2001). Expression of varied levels of many members of the subfamily (including TREK-
and KCNK7) have been demonstrated in small and medium-sized sensory DRG neurons (Dobler et al., 2007). Interestingly, inflammatory mediators and pharmacological agents are known to modulate these channels to contribute to cellular excitability mainly through setting the resting membrane potential, action potential duration and membrane input resistance (Cohen et al., 2009; Dobler et al., 2007; La & Gebhart, 2011). For example, mediators such as PGE2 induce inhibition of K_{2P} channel activity via cyclic AMP (cAMP) and protein kinase A (PKA) dependent pathways, thereby enhancing the depolarizing effects of TRPV1 beyond action potential (AP) firing threshold (Honore, 2007).

VISCERAL INFLAMMATION

A unique property of the GI tract is the presence of a low level, ongoing inflammation in the mucosa (i.e. controlled inflammation or physiological intestinal inflammation) maintained by the mucosal immune system which has developed a delicately balanced response to pathogenic organisms while co-existing with luminal resident non-pathogenic microbial and food antigens (tolerance) (Corthesy, 2007).

Under normal circumstances, the epithelial barrier formed by the single layered, tightly sealed polarized cells (i.e. intestinal epithelial cells [IECs]) allows entry of a few microbial and food antigens into specific regions of the mucosa that contain aggregates of lymphoid tissues (follicle-associated epithelia) through
specialized microfold (M-cells) cells (Al-Sadi et al., 2009; Kraehenbuhl & Corbett, 2004). These lymphoid aggregates are regularly scanned by resident immune cells such as dendritic cells, intra-epithelial lymphocytes, lamina propria mononuclear cells and regulatory T-cells. Their antigenic activation by the few non-pathogenic antigens lead to enhanced expression of anti-inflammatory factors such as TGF-β, IL-10, IL-4, and IL11 that suppresses the differentiation of T-lymphocytes into Th1 and Th2 phenotypes (Kraehenbuhl & Corbett, 2004; Liu et al., 2007). This limits production levels of pro-inflammatory cytokines like IFN-γ, IL-1, IL-6, IL-12 and TNF-α, thus leading to a state of “hyporesponsiveness or tolerance” to the luminal commensals (Fiocchi, 2001). In addition, suppression of the expression of pattern recognition receptor (necessary for T-cell activation and differentiation), and the synthesis and release of neutralizing immunoglobins (e.g. IgA) are other suggested factors contributing to the maintenance of tolerance to luminal commensals (Kraehenbuhl & Corbett, 2004).

However, injuries of any sort, including infection or damage caused by inflammation, permit the entry of increased number of pathologic organisms and other antigenic substances into the mucosa. These pathogens trigger the innate and adaptive immune cells to mount a swift but moderate level of controlled transient reaction characterized by the release of a wide variety of pro-inflammatory cytokines, including INF-γ, TNF-α, IL-1, and IL-6 from the activated immune cells, damaged tissues and affected sensory nerve fibers, targeted at eliminating the harmful agents and stimulating regeneration of damaged tissues.
to normalcy. Following this swift response, a complete resolution is most often achieved and the immune machinery turned back to the hyporesponsive state through targeted production of anti-inflammatory mediators (Vergnolle, 2008). Infrequently however, impairment of resolution may occur, which allows the process to progress into a more severe phase (Medzhitov & Janeway, Jr., 2000). At this stage, T-lymphocytes differentiate into polarized phenotypes, T helper (Th) type-1, type-2 or type-17 cells, each being unique in the type of cytokines secreted (Mosmann & Coffman, 1989). IFN-γ, IL-1, IL-6 and TNF-α are mainly secreted by Th1 cells, while IL-4, IL-5, IL-9, IL-10 and IL-13 dominate Th2 immune reactions. Th17 cells primarily secrete IL-6, IL-17 and IL-22 (Blumberg, 2009). Imbalances in Th1 (pro-inflammatory)/Th2 (anti-inflammatory) cytokine profiles characterize a number of visceral conditions. In these conditions, persistent interactions of microbial antigens with the mucosal immune system (especially in genetically susceptible individuals) induce the release of measurable levels of mediators that perpetuate the chronicity of the inflammatory cascade, and the manifestation of clinical symptoms, including pain, that characterize conditions such as IBD and IBS.

INFLAMMATORY BOWEL DISEASE (IBD)

The inflammatory bowel diseases (IBDs), comprised of Crohn’s disease (CD) and ulcerative colitis (UC), are lifelong disabling conditions characterized by alternating periods of chronic GI inflammation and remissions. As a result of the
inflammation, there is an accompanying relentless tissue destruction (Shih & Targan, 2008), along with diarrhea, rectal bleeding, abdominal pain, weight loss and in some cases extra intestinal disorders like eye and skin diseases, retarded growth and early sexual maturation in children can occur (table 1-1) (Hanauer & Present, 2003). Although the exact mechanisms associated with the development of IBD are not known, recent advances in the understanding of pathogenesis essentially recognizes important interactions between three major co-factors: environmental (e.g. microbial agents, childhood infections, smoking, diet, hygiene, population, occupation, etc.); host (defective mucosal barrier to luminal antigens and unregulated or dysregulated host immune response); and genetic susceptibility (Fiocchi, 1998; Papadakis & Targan, 1999). The complex interplay between these factors is thought to be particularly important in constantly activating the mucosal immune systems to maintain the circle of inflammatory reactions and associated tissue destructions.

Although CD and UC are generally known as the inflammatory bowel diseases, certain important differences (for example in the pattern of inflammation and cytokine profiles) exist between the two. Inflammation in CD could involve any part of the GI tract, with the ileocecal region being the most commonly affected. The lesions are characteristically transmural and may be patchy in nature; meaning that all layers of an affected gut wall are involved, with areas of ulceration separated by normal tissues (Baumgart, 2009; Loftus, Jr. et al., 2002). Recent studies suggest that the inflammatory reactions in CD are
associated with a Th1 cytokine profile characterized by the increased expression of IFN-γ, TNF-α and IL-12 (Fuss et al., 1996; Plevy et al., 1997). Unlike CD, inflammation in UC is continuous, affecting only the outer layer of the large intestine (mucosa and submucosa), and in the majority of cases the rectum. However, the Th1/Th2 cytokine profile in UC is not very clear. Analysis of transcripts for intestinal cytokines in UC patients has provided evidence for the presence of both Th-1 and Th-2 profiles (Sawa et al., 2003). In these studies, increased mRNA levels of cytokines that characterize Th-1 (i.e. IL-12, INF-γ, and TNF-α) and Th-2 (e.g. IL-4, IL-5 and IL-8) responses were detected, suggesting a role for both. However, others have suggested that the profile bears a resemblance to a modified Th-2 response (Fiocchi, 1998; Fuss et al., 1996; Fuss et al., 2004).

Studies described in this thesis employed TNF-α as a prototypical example of the mediators released in the mucosa of IBD patients, together with human supernatants produced from colonic biopsies of UC patients to investigate inflammation-induced changes in peripheral nociceptive signaling in IBD. We focused on TNF-α because it had been previously suggested to be a key mediator in the inflammatory process underlying inflammatory bowel disease (Komatsu et al., 2001; Papadakis & Targan, 2000). Support for this role comes from the dramatic results obtained from clinical trials, in which a majority of IBD patients responded remarkably well to treatment with an anti-TNF-α monoclonal antibody (Infliximab) (Baert et al., 1999) compared to those treated with placebo;
and the suppression of inflammatory processes seen in animal models of intestinal inflammation exposed to anti-TNF-α antibodies (Powrie et al., 1994).

IRRITABLE BOWEL SYNDROME (IBS)

Among the functional bowel disorders, irritable bowel syndrome (IBS) is one of the most common types. It is the most frequent disorder seen by gastroenterologists in their daily practice, accounting for more than 30% of outpatients visits (Drossman et al., 2002; Longstreth et al., 2001). IBS is a chronic condition that affects the lower gastrointestinal tract, and sufferers report recurrent abdominal pain or discomfort associated with alterations in bowel habits as major symptoms (Cain et al., 2006). However, other symptoms such as stool urgency and straining, constant feeling of incomplete voiding, and excessive flatulence have also been reported (Spinelli, 2007). Despite these reported symptoms, examination of the intestinal mucosa of IBS patients using endoscopic and routine histology methods paradoxically reveals no detectable mucosal or biochemical abnormalities (Barbara et al., 2002; De Giorgio & Barbara, 2008; Spiller, 2003).

On the basis of the most dominating symptoms, IBS can be subcategorized into: diarrhea predominant (IBS-D); constipation predominant (IBS-C); or diarrhea alternating with constipation (IBS-A) (De Giorgio & Barbara, 2008; Spinelli, 2007). In the past, making a positive diagnosis of IBS could be a daunting task for gastroenterologists due to the elusiveness of biomarkers and
heterogeneity of IBS symptoms. Now, a symptom-based diagnostic approach that allows for a more definitive diagnosis without requiring elaborate laboratory investigations was recently developed by an international team of experts on IBS (i.e. the Rome criteria) (Brandt et al., 2002; Longstreth et al., 2006). These criteria enable physicians to make a diagnosis of IBS with a reasonable certainty.

The etiopathogenesis of IBS is not fully understood. A number of mechanisms, including enhanced central perception of events occurring in the viscera, altered gastrointestinal motor function and psychosocial stressors have been proposed by many investigators as factors contributing to the development and/or exacerbation of IBS (Gwee et al., 1999). However, recent findings of mucosal immune activation (Ohman et al., 2009a; Ohman et al., 2009b) and evidence of low-grade inflammation characterized by measurable levels of lymphocytes, mast cells and enterochromaffin cells in the mucosa of both human and animal models of the disorder suggest that immune / inflammatory mechanisms could be particularly important contributing factors (Barbara et al., 2004a; Isgar et al., 1983). For example, a recent study found approximately a third of patients who experienced acute bacteria gastroenteritis following a waterborne outbreak of dysentery developed persistent IBS symptoms, giving rise to the term post-infectious IBS (PI-IBS) (Marshall et al., 2006). As a subgroup, PI-IBS has well defined IBS symptom characteristics. In these patients, symptoms occur suddenly following acute infectious self-limiting colitis.
In our studies, we modeled this infectious self-limiting colitis in mice using *C. rodentium* infection which previous studies had shown share similar properties, and causes similar colonic inflammation as human enteropathogenic *E. coli* infection (Ghaem-Maghami et al., 2001; Luperchio et al., 2000; Wiles et al., 2006). This animal model, together with supernatants obtained from colonic biopsies of human IBS patients, set the stage for the studies described in this thesis to critically examine the mechanisms underlying inflammation-induced changes in peripheral pain signaling in IBS.

**COMPARISON OF PATHOPHYSIOLOGICAL FEATURES OF IBS AND IBD**

Although IBD and IBS are characterized by common symptoms of recurrent abdominal pain and discomfort, urgency and bloating, and changes in bowel habit, important pathophysiological differences exist between the two (table 1-1) (Moloney et al., 2011; Tresca, 2009). IBS is a syndrome, and as such is diagnosed based on a group of symptoms in the presence of no obvious structural abnormalities. IBD is a group of disorders exhibiting various degrees of mucosal/tissue destructions (ulcerations), evidenced by endoscopic, histopathologic and radiological findings.

Furthermore, even though mucosal inflammation is present in both conditions, the degree to which this is present is fundamentally different. Overwhelming inflammation associated with the secretion of a huge array of inflammatory and chemical mediators (including increased levels of TNF-α,
Table 1-1: Similarities and differences between the clinical features of IBS and IBD (adapted and modified from Tresca, 2009).

### Intestinal symptoms

<table>
<thead>
<tr>
<th>Symptom</th>
<th>IBD</th>
<th>IBS</th>
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<tbody>
<tr>
<td>Abdominal pain</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Bloating/distension</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Mucus</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Persistent diarrhea</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Rectal bleeding</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Alternating diarrhea/constipation</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Possibility of malignant transformation (e.g. colon cancer)</td>
<td>x (UC)</td>
<td></td>
</tr>
<tr>
<td>Tendency to form fistulas and strictures</td>
<td>x (CD)</td>
<td></td>
</tr>
<tr>
<td>Endoscopic evidence of inflammation</td>
<td>x</td>
<td></td>
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</tbody>
</table>

### Extra intestinal symptoms

<table>
<thead>
<tr>
<th>Symptom</th>
<th>IBD</th>
<th>IBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Worsening of symptoms during menses</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Anemia</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Delayed growth and sexual maturation in children</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Eye irritations</td>
<td>x</td>
<td></td>
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<tr>
<td>Fever</td>
<td>x</td>
<td></td>
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<tr>
<td>Weight loss</td>
<td>x</td>
<td></td>
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</tbody>
</table>

### Associated conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>IBD</th>
<th>IBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary conditions</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Anxiety</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Depression</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Arthritis</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Liver complications</td>
<td>x</td>
<td></td>
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<tr>
<td>Osteoporosis</td>
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interleukin 1 beta and interleukin 6), and extensive mucosal tissue damage characterize IBD; whereas IBS is associated with a subtle elevation of inflammatory mediators secreted by hyperplastic enterochromaffin cells (5-HT), activated lymphocytes and mast cells, with no evidence of mucosal tissue damages (Gwee et al., 1999; Lee et al., 2008; Spiller et al., 2000). Clinical signs of fever, weight loss and rectal bleeding, sometimes accompanied by extra-intestinal features, seen in IBD are usually not present in IBS. A detailed list of similarities and differences of features of IBD and IBS is provided in table 1-1.

RATIONALE FOR STUDIES DESCRIBED IN THIS THESIS

Significant numbers of people, especially in developed countries such as Canada and the United States of America, are affected by IBS. This condition causes pain and suffering, which considerably impairs quality of life (Cain et al., 2006; Gralnek et al., 2000). Despite this impact, the pathophysiology of IBS remains poorly understood. Because of this poor understanding, there is lack of adequate and appropriate therapeutic agents capable of controlling pain without side effects that limit the ability of the patients to function. Currently, pain treatment in these patients is limited to the use of pharmaceutical agents (e.g. anti-spasmotics, low dose anti-depressants) whose efficacy is variable and in some cases produce non-specific effects, such as dependence, nausea and vomiting, altered cognition, drowsiness and lower levels of energy (Lembo, 2004). Therefore, discovering new peripheral mechanisms will not only improve
our understanding of the condition, but will provide novel targets for the
development of effective drugs with fewer side effects, since such agents will
target treatment to the gut.

A major challenge to proper understanding of IBS is the lack of
appropriate models, due largely to the fact that IBS likely encompass a group of
heterogeneous disorders (Mayer et al., 2001). Among these disorders, one of the
best characterized subtypes is post-infectious irritable syndrome (PI-IBS), in
which symptoms develop suddenly following infectious self-limiting bacterial
gastroenteritis (Marshall et al., 2006). However, currently available animal
models (chemical and parasitic models) employed by many investigators to study
this condition do not adequately relate to humans (Beyak et al., 2004; Krauter et
al., 2007; Lomax et al., 2007; Moore et al., 2002). For example, several of these
models (e.g. TNBS-induced colitis) induce severe mucosal inflammation and
tissue destruction in contrast to what clinical and histopathological evidence
suggest about PI-IBS (Krauter et al., 2007; Moore et al., 2002). So it is imperative
to develop models that produce features similar to those of human PI-IBS, for
example a model that could help establish the effects of transient bacterial
infection on nociceptive DRG neurons innervating the colon.

Another challenge concerning PI-IBS is that although it is a very common
condition (Drossman et al., 2002; Gralnek et al., 2000), it is not clear why most
patients who develop acute bacterial gastroenteritis do not evolve into PI-IBS.
Epidemiological studies had suggested a number of factors, including
psychological stress (Elsenbruch, 2011), but how stress factors interact with factors associated with the transient bacterial infection, and the underlying mechanisms are not clear.

There is abundant evidence for a subtle immune activation, characterized by measurable levels of inflammatory mediators, in mucosal tissues of PI-IBS patients (Gwee et al., 1999; Lee et al., 2008; Spiller et al., 2000). However, there is little known about how the mediators act to impact nociceptive signaling in these patients. Recent studies have been particularly focused on the role of proteases (Cenac et al., 2007; Gecse et al., 2008), but the mechanisms are still not clear. In addition, questions regarding the relationship of animal models to human disease are still lingering. One plausible approach to resolving this issue may be to perform corroborative studies using biopsy samples taken from the colon of patients with PI-IBS (Cenac et al., 2007; Gecse et al., 2008).

In other conditions such as IBD, traditionally recognized as chronic inflammatory conditions, the pathophysiology seems to be very different (see table 1). For instance, there is evidence of overt immune activation in mucosal tissues of IBD patients (severe inflammation and tissue destruction) characterized by elaborate secreted mediators (De & Barbara, 2008; Shih & Targan, 2008), unlike in IBS. Hence the focus on inflammatory mediators is different. Currently, there is a great deal of focus on the role of TNF-α in inflammation in IBD and the potential for its inhibitors as therapeutic agents for
this condition (Baert et al., 1999; Sommer et al., 2001), but its role in nociceptive signaling is not fully understood.

Clarifying these issues is therefore important, because insights gained will not only advance our knowledge concerning the pathophysiology of IBS and IBD, but could provide opportunities for the development of specific drugs that will lead to better management of the patients, and as such improve their quality of life.

This thesis therefore, employed self-limiting colitis induced by C. **rodentium** infection in mice and supernatants produced from human colonic biopsies of IBS patients as models to examine peripheral mechanisms underlying inflammation-induced changes in nociceptive signaling in IBS, and assessed whether an interplay exists between central and peripheral factors. Furthermore, it examined the effects of IBD inflammatory mediator milieu, including TNF-α, on nociceptive DRG neurons signaling using supernatants produced from colonic biopsies of human UC patients.

The overall hypothesis for studies described in this thesis is that mediators of inflammation would alter peripheral nociceptive signaling in patients with IBS and IBD by direct activation and/or sensitization of colonic afferent DRG neurons, and that chronic stress exaggerates the effects.
GOALS OF THIS THESIS

To test the overall hypothesis, this thesis specifically examined:

1) Whether inflammation induced by *C. rodentium* infection, a model of human *E. coli* enteritis, alters peripheral nociceptive signaling through changes in the electrical properties of colon DRG neurons and, if so determine whether the alterations are sustained after infection and inflammation had resolved. These studies are described in chapter 2.

2) Whether interactions exist between chronic stress mediators and inflammatory mediators (resulting from previous acute bacterial colitis) that underlie increased peripheral nociceptive signaling in PI-IBS. These possible interactions were examined in studies described in chapter 3.

3) Whether cysteine proteases contribute to abnormal peripheral nociceptive signaling in patients with PI-IBS. The experiments that examine such possibilities are described in chapter 4.

4) Mechanisms that underlie abdominal pain expression in patients with IBD, by studying effects of inflammatory mediators individually (e.g. TNF-α) or as a group (net effect of mediators contained in supernatants from colonic biopsies of UC patients) on nociceptive neurons. The series of studies that examined these objectives are described in chapter 5.
CHAPTER 2: CITROBACTER RODENTIUM COLITIS EVO kes POST-INFECTIONOUS HYPEREXCITABILITY OF MOUSE Nocicepti ve Colonic DRG Neurons
ABSTRACT

To investigate the possible contribution of peripheral sensory mechanisms to abdominal pain following infectious colitis, we examined whether the *Citrobacter rodentium* mouse model of human *E. coli* infection caused hyperexcitability of nociceptive colonic DRG neurons and whether these changes persisted following recovery from infection. Mice were gavaged with *C. rodentium* or distilled water. Perforated patch clamp recordings were obtained from acutely dissociated Fast Blue labeled colonic DRG neurons and afferent nerve recordings were obtained from colonic afferents during ramp colonic distensions. Recordings were obtained on day 10 (acute infection) and day 30 (infection resolved). Following gavage, colonic weights, myeloperoxidase (MPO) activity, stool cultures, and histological scoring established that infection caused colitis at day 10 which resolved by day 30 in most tissues. Electrophysiological recordings at day 10 demonstrated hyperexcitability of colonic DRG neurons (40% mean decrease in rheobase, *p* = 0.02; 50% mean increase in action potential discharge at twice rheobase, *p* = 0.02). At day 30, the increase in action potential discharge persisted (~150% increase versus control; *p* = 0.04). In voltage clamp studies, transient outward (*I_A*) and delayed rectifier (*I_K*) currents were suppressed at day 10 and *I_A* currents remained suppressed at day 30. Colonic afferent nerve recordings during colonic distension demonstrated enhanced firing at day 30 in infected animals. These studies demonstrate that acute infectious colitis evokes hyperexcitability of colonic DRG neurons which
persists following resolution of the infection and that suppression of $I_A$ currents may play a role. Together, these findings suggest that peripheral pain mechanisms could contribute to post-infectious symptoms in conditions such as post-infectious Irritable Bowel Syndrome.
INTRODUCTION

Visceral hyperalgesia, or exaggerated sensitivity to nociceptive stimuli, is often preceded by intestinal inflammation. For example, irritable bowel syndrome (IBS) (Thabane et al., 2007), a common disorder which exhibits visceral hyperalgesia, frequently occurs following an infectious gastroenteritis. Bacterial gastroenteritis is the strongest predictive factor identified to date for the development of IBS (Rodriguez & Ruigomez, 1999). Patients typically complain of abdominal pain and diarrhea-predominant symptoms, and symptoms often persist for years (Spiller, 2007). Post-infectious IBS (PI-IBS) was first described more than 5 decades ago and was best characterized following the large outbreak in Walkerton, Canada, affecting 2700 residents through contaminated water supply with Escherichia coli 0157:H7, Campylobacter jejuni, and other pathogens (Marshall et al., 2006). In this study ~30% of infected individuals developed PI-IBS and a recent meta-analysis among all available studies suggests the pooled incidence of PI-IBS is 10% (Thabane et al., 2007). Hence, PI-IBS is a common cause underlying IBS. A better understanding of the mechanisms involved could have an important impact on treatment and prevention of symptoms associated with this and related disorders.

There is considerable evidence from human studies that the abdominal pain experienced by PI-IBS patients results from dysregulation of sensory neural pathways (Kellow et al., 1991; Whitehead & Crowell, 1991). This abnormality could result from peripheral mechanisms (e.g. altered signaling in dorsal root
ganglia neurons), central mechanisms (e.g. heightened perception of pain), or possibly both (Azpiroz et al., 2007). Peripheral mechanisms may be particularly important in PI-IBS given the observations that patients have altered mucosal permeability and there is evidence for persistent low levels of inflammation following resolution of the infection (Marshall et al., 2004; Tornblom et al., 2002) but study of peripheral mechanisms in humans is limited by the lack of accessibility. The findings from animal models of chemically-induced colitis, which results in a severe transmural inflammatory process, demonstrate that during active inflammation colonic nociceptive DRG neurons are hyperexcitable. Whether these changes are representative of those which would result from an acute bacterial infection of the colon, where the severity and nature of the inflammation differs, however, is not clear. Moreover, to understand PI models such as IBS, it is particularly important to know whether changes in excitability persist after the infection and acute inflammation have resolved.

Human E. coli colitis can be modeled by the Citrobacter rodentium murine model of self-limiting colitis, providing an opportunity to examine the role of peripheral mechanisms in an animal model of bacterial infection. These bacteria attach and efface in a similar fashion to E. coli and cause a similar self-limiting inflammation (Barthold et al., 1978; Ghaem-Maghami et al., 2001; Wiles et al., 2006). Given the close resemblance of this infection to the human E. coli infection, a common cause of PI-IBS, we utilized this model to examine the possible role of bacterial infection in modulating peripheral sensory mechanisms
following resolution of the infection and overt inflammation. We used retrograde labeling to enable nociceptive DRG neurons innervating the infected region of the colon to be examined using patch clamp and afferent nerve recording techniques to address two central aims. Firstly, the excitability of the colonic neurons was examined during the acute infection to determine if inflammation induced by bacteria causes neuronal hyperexcitability, as seen with more severe chemically-induced inflammation. Secondly, neurons were studied at day 30, a time point at which previous studies have shown that infection and inflammation have resolved (Khan et al., 2006; Skinn et al., 2006), to determine if changes in excitability of the neurons persisted, and if so, what ionic mechanism may be involved.

METHODS

C. rodentium model of colitis

C57BL/6 mice (20-25 g) of either sex were obtained from Charles River Laboratories (Montreal, QC, Canada). All protocols were approved and monitored by the Queen’s University Animal Care Committee and conformed to the Guidelines of the Canadian Council of Animal Care. Mice were killed by cervical tran-section following induction of general anaesthesia using isoflurane (Baxter Corporation; Mississauga, ON, Canada) inhalation.

C57BL/6 mice were gavaged with 6.6 X 10^{11} colony forming units of C. rodentium (strain DBS 100) as previously described (Luperchio & Schauer,
2001; Mundy et al., 2005) or distilled water. Daily subcutaneous injections of 0.5 cc lactated Ringer solution were given for a week to avoid dehydration, and mice were monitored for feeding, signs of pain, and weight loss throughout the experimental period. Mice were studied at day 10 (active infection) and day 30 (after resolution of infection).

At day 3 or day 20 after C. rodentium or distilled water were gavaged, surgeries were performed on mice to label colonic DRG neurons with Fast Blue fluorescent retrograde label (Cedarlane Laboratories; Hornby, ON), as previously described (Beyak et al., 2004; Kayssi et al., 2007). The different time points were chosen to ensure Fast Blue labeling was not lost by day 30. Briefly, mice were anesthetized with intra-peritoneal injection (0.1 ml/10 g body wt) of a combination of ketamine (Pfizer; New York, NY) (18.75 mg/ml) and xylazine (Bayer; Etobicoke, ON) (1.25 mg/ml). A midline laparotomy was then performed to expose the descending colon. Under a dissecting microscope, 17 mg/ml Fast Blue was injected (10 μl per injection) into 5-10 sites along the colon wall. The colon was replaced and the wound sutured with 4.0 silk. Buprenex (Buprenorphine 0.25 μg/g body wt I.P.) was given to control post-operative pain. Animals were allowed to recover on a warm blanket, monitored for signs of pain and were given free access to food and water.
**Cell preparation**

At day 10 or day 30 following *C. rodentium* gavage, DRG neurons were acutely dissociated from T9 to T13 ganglia or from T1 to T3 (for studies with non-labeled neurons from a region of the spinal cord which does not innervate the colon), as previously described (Moore *et al.*, 2002; Stewart *et al.*, 2003). Briefly, tissue was incubated in collagenase (Worthington; Lakewood, NJ) (1 mg/ml) and dispase (Roche; Indianapolis, IN) (4 mg/ml) for 15 min at 37 °C, triturated with a fire-polished Pasteur pipette. Dispersed neurons were suspended in DMEM (pH 7.2 - 7.3) containing 10% fetal bovine serum, 100 U/ml Penicillin, 0.1 mg/ml Streptomycin, and 2 mM glutamine, plated on PureCol-coated (60 μl/ml) (Inamed Biomaterials, Fremont, CA, USA) cover slips and incubated overnight in a humidified incubator at 95% O2 and 5% CO2 until retrieval for electrophysiological studies.

**Electrophysiological recordings**

Perforated patch clamp experiments were performed in current or voltage clamp modes at room temperature. Fast Blue labeled neurons were identified by their bright blue fluorescence under brief exposure to ultraviolet light, using a U-MWIG2 filter. Only small neurons (≤ 40 pico-Farad (pF); i.e. large Fast blue labelled neurons were not studied) were studied because these neurons have been shown to display properties associated with nociceptors (i.e. capsaicin sensitivity, TTX-resistant action potentials) (Akopian *et al.*, 1996; Beyak & Vanner,
Signals were acquired using an Axopatch 200B amplifier and digitized with a Digidata 1322A A/D converter (Axon Instruments, San Jose, CA). Signals were low-pass filtered at 5 kHz, acquired at 20 kHz, stored and analysed using Clampfit 10.0 (Axon Instruments). Capacitive transients were corrected using analog circuitry. Inclusion criteria for analysis of cells in current clamp mode were resting membrane potentials more negative than -40 mV and overshooting action potentials with a hump on the falling phase.

Perforated patch recordings were obtained using Amphotericin B (240 μg/ml) from Sigma (St. Louis, MO) as described previously (Cooper et al., 1990; Rae et al., 1991). Due to the compound’s photosensitivity and rapid rate of breakdown upon exposure to light, mixed Amphotericin B solutions were replaced every 2-3 hours. Solutions; (in mM) extracellular solution: 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, 10 D-glucose, pH 7.4. Pipette solution: 110 K-gluconate, 30 KCl, 10 HEPES, 1 MgCl₂, 2 CaCl₂, pH 7.25. The liquid junction potential was taken to be 12 mV, and corrected for. For isolation of potassium currents in voltage clamp recordings the following solutions were used; Extracellular: 140 NMDG, 4 KCl, 1.8 HEPES, 1 D-glucose, 1 CaCl₂, 1 MgCl₂, pH 7.4. Pipette solution: 110 K-aspartate, 30 KCl, 10 EGTA, 10 HEPES, 2 Na₂-ATP, 1 MgCl₂, pH 7.2. The liquid junction potential was calculated to be 7.3 mV and corrected for. I_A and I_K currents were separated based on their biophysical properties, as previously described (Stewart et al., 2003). Briefly, total K⁺ current
was elicited from a holding potential of -100 mV with depolarizing pulses in 10 mV increments between -90 mV to +50 mV. These steps were repeated with \( I_A \) inactivated by using a holding potential of –60 mV, and the traces subtracted to provide \( I_A \). \( I_A \) inactivation properties were studied using a two pulse protocol as previously described (Stewart et al., 2003); a 1 s pre-pulse varying between -120 and 0 mV, followed by a 400 ms test pulse of +50 mV. Currents during the test pulse were normalized and plotted against the conditioning potential and fitted with the Boltzmann function: 

\[
\frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp\left(\frac{V_{50} - V_m}{k}\right)}
\]

where \( I \) is the current, \( I_{\text{max}} \) is the maximal current, \( V_{50} \) is the membrane potential for half-activation, \( V_m \) is the command potential, and \( k \) is the slope factor.

**Recording of afferent nerve activity in response to intra-luminal distension pressure**

Experiments were performed on *C. rodentium* infected or sham animals at day 10 or 30. Animals were killed by cervical tran-section and their abdominal cavity opened. The distal colon and rectum were dissected from the pelvis with attached mesenteric nerves and placed in a Sylgard-lined organ chamber which was continually perfused with oxygenated Krebs solution (in mM: NaCl 118.4; NaHCO\(_3\) 24.9; CaCl\(_2\) 1.9; MgSO\(_4\).7H\(_2\)O 1.2; KH\(_2\)PO\(_4\) 1.2 and D-glucose 11.7) at a flow rate of ∼8 ml/min and maintained at 33-34 °C. Proximal and distal ends of the bowel were securely attached to an input and outlet port. The input port was connected to a perfusion syringe pump, which allowed continuous intraluminal
perfusion of Krebs solution through the segments (0.2 ml/min) or periodic distension when closed. Intra-luminal pressure was recorded via a pressure amplifier (NL 108, Digitimer, UK) connected in parallel with the input port. The mesenteric bundle was pinned out to the base of the chamber and a mesenteric nerve was dissected out from the bundle and drawn into a suction electrode. The electrical activity was recorded by a Neurolog headstage (NL 100, Digitimer Ltd, UK), amplified (NL104), and filtered (NL125 band pass 200-3000 Hz) and acquired (20 kHz sampling rate) to a PC through a Micro 1401 MKII interface running Spike 2 software (Cambridge Electronic Design, UK). The preparation was stabilized for 30 minutes and was distended to an intra-luminal pressure of 60 mmHg with closure the outlet port. Only afferent discharge responses to ramp distensions that were stable and reproducible (at least 3 times 10 min apart) were analyzed. The third response was analyzed in each case.

**Evaluation of colonic inflammation**

**Histopathology assessment:** At day 10 or day 30 post-infection with *C. rodentium* or control distilled water, colon samples were taken for pathological assessment. Full thickness colonic tissue samples for histology assessment were fixed in 10% neutral buffered formalin. Following paraffin embedding, 5 μm thick sections (3 cross sections from each colon specimen/block) were cut and stained with hematoxylin and eosin (H&E). Slides were reviewed by a pathologist (DH) who was blinded to study group origin. *C. rodentium* or sham treated colonic
tissues were also stained with toluidine blue stain to identify mast cells (Tuna et al., 2006).

Assessment of colonic pathology utilized a semiquantitative colitis histology scoring system that evaluated and scored for inflammation and crypt height. Inflammation score (score range 0-9) was determined by assessment of a) presence and intensity of mononuclear cell infiltrate (0-none; 1-mild; 2-moderate; 3-severe), b) presence and intensity of neutrophilic cell infiltrate (0-none; 1-mild; 2-moderate; 3-severe), c) maximum depth of mural inflammatory infiltrate (0-none; 1-mucosa; 2-mural), and d) mucosal lymphoid hypertrophy (0-absent; 1-present). Mean crypt height (in microns) was calculated for each specimen from individual measurements (at least 5; most cases greater than 15) of the lengths of all well oriented crypts on each specimen slide using an ocular micrometer.

**Myeloperoxidase enzyme (MPO) assay**: Full thickness distal colonic tissues were isolated from *C. rodentium* or control saline gavaged mice and assayed for enzyme activity, as previously described (Bradley et al., 1982). Isolated tissues were cut open along the mesenteric border, rinsed with normal saline to remove fecal matter and weighed. 200 mg tissue samples were homogenized for 15 seconds in 1 ml HTAB buffer (5g Hexadecyltrimethylammonium Bromide in 1 L potassium phosphate buffer; Sigma) using a polytron tissue homogenizer. Following homogenization, additional buffer was added to each tube to give 1 ml of buffer per 50mg tissue.
The tubes were vortexed and about 1 ml of supernatant descanted into an eppendorf tube and centrifuged for 2 minutes at maximum speed in a benchtop eppendorf centrifuge. Enzyme activity was then determined by adding 7 μl of the supernatant to 200 μl of O-dianisidine buffer solution (16.7 mg O-dianisidine dihydrochloride; Pfaltz & Bauer, Inc., Waterbury, CT, USA, in 5 mmol/L phosphate buffer containing 0.005% H₂O₂) in a 96 well plate. The plates were read immediately using a Microtiter plate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA). Changes in absorbance at 460 nm were measured 3 times at 30 seconds interval and values expressed as units of MPO activity per mg tissue sample, where one unit of MPO is defined as that which degrades 1 μmol of hydrogen peroxide per minute.

**Statistical analysis**

Results are tested with unpaired Student’s t-test or 2-way ANOVA with Bonferroni post-hoc test and the significance was set at p < 0.05. Data are expressed as means ± SEM. Fitting of data was done using the Boltzmann equation fit function in Origin 6.0 (Microcal). Voltages of half-activation (V₅₀) and slope factors (k) were obtained from the individual Boltzmann curve fits. The mean firing frequency (impulses (imp s⁻¹)) were measured with a time constant of 10 s. Firing rates were measured using a bin width of 1 s while the stimulus response-curves for whole nerve activity were constructed for 5 mmHg increments using customized script program (CED, Cambridge, UK).
RESULTS

Effects of *C. rodentium* induced colitis at day 10 on neuronal excitability

All animals were monitored daily and most infected animals (85-90%) exhibited signs of a brief illness for 2-3 days following gavage (e.g. decreased activity) but recovered quickly. A small number of animals were sicker for a more extended period but by 7-10 days there were no observable differences from the controls. There was a 2-3% mortality rate which occurred within the first 3 days following infection.

The excitability of Fast Blue labeled DRG neurons was determined by measuring differences in membrane potential, rheobase, number of action potentials at twice rheobase, and input resistance (Figure 2-1), as previously described (Beyak *et al.*, 2004; Malykhina *et al.*, 2006; Moore *et al.*, 2002). Individual cells in all current clamp experiments were obtained from ≥ 5 animals (total number = 33 animals) in each series of experiments. In current clamp mode, recordings were obtained from small Fast Blue labeled DRG neurons from animals infected with *C. rodentium* (n = 27 cells), and controls (n = 22 cells) (Figure 2-1A & B). At day 10 (Figure 2-1B), when infection and inflammation were established, the mean rheobase was significantly decreased (mean = 37.8 vs. 63.6 pA; p = 0.02), compared to controls. The mean number of action potentials at twice rheobase was also significantly increased in the *C. rodentium* group compared to controls (4.3 vs. 2.8 respectively; p = 0.02). The mean resting membrane potential was slightly lower in the *C. rodentium* group
compared to controls (mean -54.4 ± 0.4 mV vs. -57.9 ± 0.7 mV; p = 0.0001). In contrast, the mean input resistance did not differ between the two groups.

The excitability of DRG neurons which did not innervate the inflamed colon (from ganglia in T1-T3 at day 10 in C. rodentium infected mice and controls) was also compared. No difference in the rheobase or number of action potentials at twice the rheobase were observed between the infected (n = 11 cells) and non-infected animals (n = 15 cells) (rheobase = 66.4 ± 9.2 pA for C. rodentium infected and 69.3 ± 8.1 pA for control cells, respectively; number of action potentials at twice rheobase = 2.3 ± 0.6 for C. rodentium infected and 2.1 ± 0.4 for controls).

**Neuronal hyperexcitability persists after resolution of acute infection**

Previous studies suggest that infection and inflammation resolve by day 30 following oral inoculation with C. rodentium (Khan *et al.*, 2006; Skinn *et al.*, 2006). Hence, we used the day 30 time point to examine whether changes in neuronal excitability were still evident following resolution of the infection. At this time point, we found that the mean number of action potentials at twice rheobase were still markedly increased (mean = 4.1, n = 22 cells vs. 2.5, n = 16 cells; p < 0.04) compared to controls (Figure 2-1 B). These changes were similar to those observed at day 10, suggesting these changes were sustained following their induction with the initial infection. In contrast, other parameters of excitability
Figure 2-1 Effects of *C. rodentium* induced colitis on excitability of colonic DRG neurons. (A) Representative current clamp trace of action potential elicited at rheobase and two times rheobase from labeled neurons isolated from *C. rodentium* treated and control animals. The amount of current needed to elicit an action potential is significantly lower in labeled *C. rodentium* neurons (10 pA) compared to control neurons (50 pA). Furthermore, the *C. rodentium* neurons fire significantly more action potentials at 2x rheobase compared to control neurons. Action potential tracings from control neurons are shown in upper panel and those from *C. rodentium* treated neurons are shown in lower panel. A 500 ms depolarising current pulse was used to elicit the action potentials. Insert shows one of the action potentials using expanded time scale to exhibit the characteristic hump on the falling phase typical of nociceptive neurons. (B) Data summarizing changes in electrophysiological properties of nociceptive DRG neurons during acute (day 10) and following resolution of infection (day 30). *C. rodentium* induced colitis caused a significant reduction in rheobase, significant increase in the mean number of action potentials at 2x rheobase and a significant reduction in the mean resting membrane potential in labeled neurons isolated from *C. rodentium* treated (gray bars) animals during acute infection compared to matched controls (dotted white bars). The mean input resistance did not change significantly. Data represents mean ± SEM. *P < 0.05; NS = not significant. At day 30, neurons from *C. rodentium* treated (black bars) animals fired significantly more action potentials at 2x rheobase compared to controls (white bars) whereas, the mean rheobase, resting membrane potential and input resistance returned to comparable control values. Data represents mean ± SEM. *P < 0.05.*
(rheobase and resting membrane potential) returned to values similar to that observed for controls (Figure 2-1B).

**Monitoring of *C. rodentium* infection and colitis**

Four parameters of infection and inflammation were monitored in samples obtained from animals used in the electrophysiological experiments. Fecal samples, colon weight, MPO activity and histological scores were studied to confirm the evolution of infection and inflammation. Fecal pellets were cultured for bacteria at days 10, 15, 20, and 30 (Table 2-1). *C. rodentium* organisms were absent by day 15.

**Table 2-1: *C. rodentium* enumeration in fecal samples**

<table>
<thead>
<tr>
<th></th>
<th>Day 10</th>
<th>Day 15</th>
<th>Day 25</th>
<th>Day 25</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (CFU (g pellet)^−1)</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td>(n = 8)</td>
<td></td>
</tr>
<tr>
<td>Citrobacter (CFU (g pellet)^−1)</td>
<td>5.0 x 10^5 ± 461661</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td>(n = 14)</td>
<td></td>
</tr>
</tbody>
</table>

During active infection (day 10), the mean wet weight per length of colon from the *C. rodentium* group (n = 13 colons) was almost 70% greater compared to uninfected controls (n = 9 colons; p < 0.0002). Following resolution of infection (day 30), this had returned to comparable control levels (n = 14 colons for *C. rodentium* and 12 colons for control groups) (Figure 2-2A). Similarly, MPO
activity was markedly increased in colonic tissues at day 10 (n = 5 colonic tissues for *C. rodentium* and 6 colonic tissues for control groups) and values had returned to control levels when measured at day 30 (n = 7 colonic tissues for *C. rodentium* and 6 colonic tissues for control groups) (Figure 2-2B).

Although previous studies have suggested that inflammation induced by *C. rodentium* had resolved by day 30 (Khan *et al.*, 2006; Skinn *et al.*, 2006) and this was also suggested by our MPO data and wet weight measurements (Figure 2-2), we also conducted a careful histological assessment by a GI Pathologist, who was blinded to the presence or absence of infection, to provide a sensitive measure of subtle inflammation at this time point (Figure 2-3A & B). Microscopic damage scores demonstrated a marked increase in the inflammation composite score (range 3-6; p < 0.0001) and crypt hyperplasia at day 10 in colons infected *C. rodentium* compared to controls (Figure 2-3A & B). At day 30, most tissues could not be distinguished from controls, but our detailed histological scoring system suggested there was a mild residual inflammation in some animals (control range 0-2, *C. rodentium* 0-3; see Figure 2-3B). Unlike the tissues from infected animals at day 10 where neutrophils were prominent, tissues from day 30 animals rarely exhibited neutrophils in keeping with the MPO measurements. Although subtle inflammation can be a feature of IBS (Marshall *et al.*, 2004; Tornblom *et al.*, 2002), we conducted a post-hoc evaluation of neurons from tissues whose histological scores were in the same range as controls (i.e. histological scores 0) to eliminate any possible contribution this low level...
Figure 2-2 Monitoring of *C. rodentium* colitis. (A) Scatter plot of colonic wet weight during active infection (day 10) (n = 13 animals for *C. rodentium* and 9 animals for control); *** p < 0.0002 and following resolution (day 30) of infection (n = 7 animals for both *C. rodentium* and control). The lines demonstrate the mean ± SEM for each treatment group. (B) Increased MPO activity was detected during acute infection (day 10) in tissue samples from *C. rodentium* infected animals (black bars) compared to controls (white bars), whereas levels were comparable to control values on resolution of the infection (day 30). Data represents mean ± SEM values; ** P < 0.005.
Sustained hyperexcitability of colonic DRG neurons following resolution of *C. rodentium* induced colitis. (A) Representative photomicrographs of H&E staining of colonic tissue sections illustrating normal histology of control tissues, increased inflammation and crypt height during active infection (day 10) and return to near normal colonic mucosal histology on resolution of infection (day 30). Scale bar = 100 μm. (B) Microscopic histologic colitis scores determined by a pathologist blinded to the treatments. Mean composite scores of inflammation are shown in upper panels and mean crypt heights are shown in the lower panels for active infection on day 10 (left panels) and resolved infection on day 30 (right panels). For day 30 colons, (T) = all colons and (NI) = non-inflamed colons only, based on microscopic inflammation scores. Values show a marked increase in inflammation at day 10 which had resolved in most colons by day 30 although some still showed subtle evidence of inflammation. ***P < 0.0001, **P < 0.005. (C) Summary data illustrating persistent hyperexcitability of nociceptive DRG neurons following resolution of infection (day 30). Neurons from *C. rodentium* treated animals fired significantly more action potentials at 2x rheobase compared to control. *P < 0.05. The mean rheobase was lower, but not significant in the *C. rodentium* treated neurons compared to controls.
inflammation might have in the measured level of excitability. Control recordings were obtained from animals in the same batch (i.e. each animal in a batch of 6 had a matched control to minimize for biological variability between batches of animals). This post-hoc analysis of neurons from “non-inflamed” tissue also showed a 2-3 fold increase in numbers of action potentials at twice the rheobase (Figure 2-3C), demonstrating that neurons innervating tissues considered to be non-inflamed by a GI Pathologist, remained hyperexcitable.

We also examined mast cell numbers at day 10 and day 30 using Toluidine blue. We found small numbers of mast cells at day 10, largely in the extra-mucosal bowel wall (Figure 2-4; n= 5 colons at each time point) and only a rare mast cell was evident at day 30. Only a very small numbers of mucosal mononuclear cells were observed at day 30 and these did not differ from controls.

**Suppression of $K^{+}_V$ currents contributes to sustained hyperexcitability at day 10 and 30**

The finding at days 10 and 30 of a sustained increase in the number of action potentials at twice the rheobase could suggest that $I_A$ currents were suppressed given their prominent role in regulating action potential discharge (Amberg *et al.*, 2002; Connor & Stevens, 1971; Tierney & Harris-Warrick, 1992). Previous studies of acute visceral inflammation have shown that suppression of $I_A$ currents is a common mechanism underlying DRG neuronal hyperexcitability.
Figure 2-4  Assessment of mast cell numbers at day 10 and 30 following C. rodentium infection. (A) Representative photomicrographs of toluidine blue stained colonic tissue sections illustrating intact mast cells during active infection (day 10) and following infection resolution (day 30). Scale bar = 100 μm. Insert image shows cytoplasmic granular staining of individual mast cells (scale bar = 10 μm). (B) Summary of data (n=3 sections from 5 colons) showing numbers of mast cells stained per section of colon at day 10 and day 30. Control samples from day 10 and 30 were combined. P = 0.09 comparing control and day 10 tissues (Mann-Whitney test).
during active inflammation but it is unknown whether these changes might persist in non-inflamed tissues (Stewart et al., 2003; Tan et al., 2006). Therefore, we employed voltage clamp electrophysiological methods to determine whether modulation of these currents could play a role in the sustained excitability at days 10 and 30. Individual cells were obtained from \( \geq 5 \) animals (total number = 28) for each series of voltage clamp experiments. Using voltage clamp protocols, as previously described (Beyak & Vanner, 2005; Stewart et al., 2003), \( I_A \) and \( I_K \) currents were separated based on their biophysical properties, as shown in Figure 2-5A. DRG neurons from animals previously infected with \( C. \) rodentium exhibited a > 120% and a 36% decrease in peak current density of \( I_A \) at day 10 (\( n = 15 \) cells) and day 30 (\( n = 14 \) cells) respectively, \( (p < 0.03) \) compared to control neurons (\( n = 11 \) cells at day 10, \( n = 12 \) cells at day 30) (Figure 2-5B and C). At day 10, neurons from \( C. \) rodentium infected animals also exhibited a 35% suppression of \( I_K \) peak current density (\( n = 15 \) cells) compared to control neurons (\( n = 11 \) cells), whereas, there was no significant difference in the \( I_K \) peak current density between the two groups at day 30 (\( p = 0.16) \).

The current-voltage relationship for the isolated currents demonstrated decreased currents in the \( C. \) rodentium animals near the resting membrane potential, suggesting these effects were active in the physiological range of membrane potentials (Figure 2-6A and B left panel). The voltage dependencies of activation of both \( I_A \) and \( I_K \) currents in neurons from \( C. \) rodentium infected mice and controls were examined using a single pulse protocol (see methods).
Figure 2-5 Effects of *C. rodentium* colitis on isolated voltage gated Kv currents. (A) Representative voltage clamp traces showing outward voltage gated K⁺ current families separated biophysically by manipulating the holding potential. Currents were generated using a 10 mV voltage step protocol (illustrated in inset) from -90 mV to +50 mV. At holding potential of -100 mV (left), two currents were evident, a transient inactivating A-type current (Iₐ) and sustained non-inactivating Iₖ type currents. At a holding membrane potential of -60 mV, Iₐ current was significantly inactivated such that only the sustained component Iₖ is elicited (middle) and subtraction of the sustained from the total current yielded Iₐ (right). (B) Summary of peak current density for Iₐ and Iₖ respectively. Current densities were obtained by normalizing the measured amplitudes of the peak transient components of the isolated currents to the cell capacitance. At the height of inflammation (day 10), *C. rodentium* induced colitis caused significant suppression of both Iₐ and Iₖ current densities (black bar) compared to controls (white bar). (C) Following resolution of infection (day 30), the reduction in Iₐ current density was sustained in neurons from *C. rodentium* infected animals (black bar) compared to controls (white bar). There was no significant difference in Iₖ current density between the two groups of neurons. Data represents mean ± SEM. *P < 0.05.
rodentium induced colitis did not alter the voltage dependency of activation of either current. At day 10, the values for voltage of half maximal activation $V_{1/2}$ of $I_A$ in $C. \text{rodentium}$ and controls were $-6.7 \pm 1.3$ mV and $-10.7 \pm 1.3$ mV, with a slope factor $k$ of $21.3 \pm 1.4$ and $20.3 \pm 1.4$, respectively (Figure 6A). The $V_{1/2}$ of $I_K$ were $-13.2 \pm 1.0$ mV and $-14.0 \pm 0.9$ mV $p = 0.7$, with a $k$ of $16.1 \pm 1.0$ and $13.2 \pm 0.8$, respectively (Figure 2-6A). At day 30, $V_{1/2}$ values of $I_A$ in $C. \text{rodentium}$ and controls were $-15.4 \pm 1.8$ mV and $-14.1 \pm 1.3$ mV, with a $k$ of $15.3 \pm 1.7$ and $14.1 \pm 1.3$, respectively (Figure 2-6B).

The voltage dependency of inactivation of $I_A$ and $I_K$ currents was examined using a double pulse protocol (see methods). At day 10, the half maximal value for inactivation of $I_A$ and $I_K$ in $C. \text{rodentium}$ neurons shifted significantly to the left compared to controls ($I_A = -76.1 \pm 0.4$ mV and $-65.8 \pm 0.5$ mV respectively, $p = 0.0001$; $I_K = -54.6 \pm 0.4$ mV and $-49.2 \pm 0.1$ respectively, $p = 0.001$) with $k$ of $8.6 \pm 0.4$ and $9.5 \pm 0.4$ for $I_A$; $7.2 \pm 0.4$ and $7.5 \pm 0.1$ for $I_K$, respectively (Figure 2-6B). At day 30 only $I_A$ voltage dependency of inactivation (Figure 2-6B) was examined since we did not observe any $I_K$ current suppression at this time. The half maximal value for inactivation of $I_A$ in $C. \text{rodentium}$ neurons was also shifted significantly to the left (as in day 10) compared to controls ($-84.0 \pm 0.8$ mV and $-74.0 \pm 0.7$ mV respectively; $p = 0.0001$) with $k$ of $11.3 \pm 0.7$ and $10.41 \pm 0.6$, respectively (Figure 2-6B). This sustained hyperpolarizing shift ($\sim 10$ mV) implies that neurons from the $C. \text{rodentium}$ treated animals have fewer $I_A$ channels.
available at or near the resting membrane potential, leading to increased excitability and hence the increased spike frequency.

**Colonic afferent fibers displayed increased mechanical discharge following *C. rodentium* infection at day 30**

We used an *in vitro* model to investigate changes in visceral mechanosensitivity of colonic afferents from *C. rodentium* infected and control animals. Experiments were performed with repeated ramp distensions of the bowel, at day 10 or day 30 post-infection. We found no significant difference (unpaired t-test, p < 0.05) in whole nerve spontaneous activity (duration = 100s) between control and infected animal preparations at day 10 (control 18.13 ± 4.54 spikes/s; infected 19.84 ± 3.84 spikes/s) or day 30 (control 23.05 ± 10.75 spikes/s; infected 30.01 ± 9.38 spikes/s). No differences in the afferent discharge in response to ramp distensions (60 mmHg) were observed between control (n = 10, one preparation per animal) and *C. rodentium* infected (n = 10, one preparation per animal) animals at day 10 (Figure 2-7). However, the afferent discharge at day 30 post-infection (n = 9, one preparation per animal) was significantly augmented compared to control (n = 10, one preparation per animal) (Figure 2-7; P < 0.001 two-way ANOVA).
Figure 2-6 Effect of *C. rodentium* induced colitis on the current – voltage relationship and the steady-state activation and inactivation of $I_A$ and $I_K$ at the height of infection (day 10) and on resolution (day 30). (A) Correlation of the current – voltage relationship for $I_A$ (upper left panel) and $I_K$ (middle left panel) showing significantly decreased currents by *C. rodentium* colitis near the resting membrane potential. Upper and middle central panels illustrate the steady-state activation curves for $I_A$ and $I_K$ from control and infected animals. Means ± SEM of normalized conductance ($G/G_{max}$) were plotted against membrane potential. Data were fitted using Boltzmann function, and continuous lines show best fits. The upper and middle right panels show the steady-state inactivation curves for both currents in neurons from *C. rodentium* infected animals and controls. The curves were obtained by plotting the normalized test current amplitudes against conditioning pre-pulse potentials. *C. rodentium* induced colitis caused a leftward shift in the steady-state inactivation curves for both $I_A$ (upper right panel) and $I_K$ (middle right panel). (B) At day 30, *C. rodentium* induced colitis also significantly decreased $I_A$ currents near the resting membrane potential (lower left panel). The steady-state activation kinetics of $I_A$ was not altered (lower middle panel). However, the steady-state inactivation curve for $I_A$ currents (lower right panel) in neurons from *C. rodentium* treated animals shifted to the left (hyperpolarizing shift) when compared to controls.
Figure 2-7 Assesment of visceral mechanosensitivity. (A) Representative trace showing pelvic nerve afferent activity (Spikes s⁻¹) in response to ramp distension in 30 days control or 30 day infected mice. Top traces: Ramp distension; Second trace: Afferent activity rate; Bottom trace: Raw recording of whole nerve activity. (B) Pressure-response profiles of multi-unit activity from 10 and 30 days controls or infected animals. The pressure-response profile was significantly augmented at day 30 compared to controls (p < 0.001 for all measurement points, two-way ANOVA with Bonferroni correction). Left: day 10 animals (n=10) right: day 30 animals (n=9). Data are means ± SEM per treatment group.
DISCUSSION

This study examined whether peripheral sensory mechanisms may play a role in the abdominal pain which can persist following the resolution of infectious colitis, such as found in PI-IBS. Fast Blue retrograde labeling was used to identify the DRG neurons innervating the inflamed colon and small neurons (<40 pF) were examined because numerous studies have shown they exhibit properties of nociceptors (Beyak & Vanner, 2005; Moore et al., 2002; Yoshimura & de Groat, 1999). We found that colitis resulting from C. rodentium infection for 10 days induced marked hyperexcitability of DRG neurons but had no effect on DRG neurons which did not innervate the colon. Moreover, at day 30, when histological and immune markers demonstrated the infection had resolved in the colon, colonic neurons still exhibited evidence of hyperexcitability. These findings support a role for peripheral mechanisms in the genesis of abdominal pain in conditions where symptoms persist after the inflammation has resolved, such as PI-IBS.

E. coli enteritis is one of the common causes of PI-IBS (Smith & Bayles, 2007) but the effects of bacterial induced colitis on the properties of intestinal DRG neurons has not been previously examined. We utilized C. rodentium because it is widely recognized as a surrogate model of human E. coli infection given that is has similar attachment and effacement properties and induces a self-limiting colitis (Luperchio & Schauer, 2001; Mundy et al., 2005). After 10 days of infection, we found that the resulting inflammation induces
hyperexcitability of colonic DRG neurons, given the significant decrease in rheobase and increase in action potentials discharge at two times the rheobase. In contrast to this bacterial model of colitis, most previous studies of the effects of inflammation on visceral sensory neurons have employed models of chemical inflammation, which tends to produce a more severe inflammatory response. Despite these differences in the degree and nature of the inflammation, our electrophysiological findings were similar to those described in the models of chemical inflammation, confirming that significant changes in neuronal excitability can occur in the absence of severe transmural inflammation (Krauter et al., 2007; Lomax et al., 2007). One important difference was that we did not observe significant increases in input resistance, a common finding in the studies of chemical inflammation (Beyak et al., 2004; Moore et al., 2002). Although we did not explore all of the ionic mechanisms underlying this acute hyperexcitability using voltage clamp studies because this was beyond the scope of this study, this finding may suggest that the relative contribution of varying ion channels involved may differ between these models of inflammation.

If peripheral mechanisms are to play a role in the pain associated with conditions where symptoms persist after the initial inflammation has resolved, such as PI-IBS, the hyperexcitability must be sustained after the infection has resolved or be “re-primed” for example, through periodic T-cell activation. At day 30, after the C. rodentium infection had resolved, we found that the neurons remained hyperexcitable, supporting the concept that peripheral mechanisms
can contribute to the genesis of abdominal pain after infection. A recent study of a parasitic infection in the small intestine has also shown that following resolution of infection, hyperexcitability of DRG neurons persists, lending further support to the concept that peripheral mechanisms can play a role in post-infectious abdominal pain (Hillsley et al., 2006;Keating et al., 2008). This parasitic model of jejunitis is a mast cell-dependent model (Barbara et al., 2007) as opposed to the predominantly T-cell dependent mechanisms underlying C. rodentium and E. coli (Shiomi et al., 2010;Symonds et al., 2009). Thus, it appears multiple immune pathways can induce these sustained changes in neuronal function observed in our study.

Interestingly, we did find evidence that some tissues exhibited low levels of inflammation at this late time point (i.e. day 30). However, when these were excluded from the analysis, there was still evidence of neuronal hyperexcitability (see Figure 3). Nonetheless, clinical studies of PI-IBS have shown that activated T-cells and/or mast cells may also be contributing to visceral hyperalgesia following resolution of the colonic infection (Barbara et al., 2007;Hansen et al., 2005;Spiller, 2003). We found small numbers of intact mast cells in infected tissues at day 10. Previous studies have shown significant elevation in tissue proteases in this model during this acute infection, implying significant mast cell degranulation (Hansen et al., 2005). Intact mast cells were very rare at day 30 but we cannot exclude that low levels of degranulation also occurred. Changes in serotonin signaling from colonic enterochromaffin cells in
patients with IBS have also been described (Mawe et al., 2006). Sustained hyperexcitability of DRG neurons in this setting, as found in the current study, suggests that cytokines, serotonin and/or mast cell proteases would evoke enhanced neurotransmission in nociceptive neurons compared to neurons which had not previously been exposed to an acute infection.

Multiple ionic mechanisms have been implicated in the DRG neuronal hyperexcitability observed in models of visceral inflammation (Beyak et al., 2004; Beyak & Vanner, 2005; Kayssi et al., 2007). We focused on changes in the $I_A$ and $I_K$ currents because our major finding at day 30 was the increase in action potential discharge numbers, which implied $I_A$ currents may be involved. $I_A$ has been shown to be a major current in the regulation of spiking frequency in various cell types, including neurons (Bardoni & Belluzzi, 1994) and endocrine cells (Mei et al., 1995). Changes in these currents have been implicated in most models of chemical inflammation (Beyak et al., 2004; Beyak & Vanner, 2005; Yoshimura & de Groat, 1999) but their properties have not been studied previously in models following resolution of the inflammation. We found evidence for suppression of $I_A$ currents at day 30, which would contribute to the increased firing rate observed following resolution of the infection. It is unknown whether these persisting changes reflect altered transcription of the channel or modulation of existing channels within the membrane. It is also likely that other $K^+$ channels are involved, although the absence of changes in input resistance may suggest that changes in leak currents play less of a role than suggested in
other models of inflammation. Given the evidence that visceral inflammation can also modulate other channels (Beyak et al., 2004; Stewart et al., 2003), further studies are needed to determine if Na\(^+\), Ca\(^{2+}\) and/or TRP channels may also be altered by this infection (De Schepper et al., 2008).

We studied the physiological implications of the sustained neuronal hyperexcitability by examining the colonic afferent nerve response to ramp distensions of the colon in C. rodentium infected animals. These studies provide evidence for peripheral afferent hyperexcitability in the day 30 infected animals and thus support the concept that persisting changes on voltage gated ion channels in nerve terminals of DRG neurons contribute to altered sensory regulation in this post-infectious state. We were unable to perform single fiber analysis due to the high frequency response and it is possible that additional differences may exist between specific fiber types (i.e. including those at day 10 where overall differences were not seen). Recent studies suggest important differences may exist between specific fiber types and pelvic compared to splanchnic nerves in response to inflammation (Hughes et al., 2009b; Lynn et al., 2008). A previous study of intestinal afferents has also shown a correlation between mechanosensitivity and neuronal hyperexcitability (Keating et al., 2008), although the current study is the first to identify one of the candidate ion channels (i.e. \(I_A\) currents). Not all models of inflammation however have provided evidence of enhanced mechanosensitivity following resolution of the inflammation (Aerssens et al., 2007; Coldwell et al., 2007; Larsson et al., 2006). There are no
available electrophysiological correlates in these studies concerning the excitability parameters of the DRG neurons and thus, it is unclear whether differences reflect the nature of the inflammation (Keating et al., 2008) or other contributing factors, for example 5-HT signaling or stress mediated effects that may persist following resolution of the inflammation. Indeed, there is evidence that C. rodentium infected animals showed increased levels of stress acutely (Lyte et al., 2006) and our routine handling of the infected animals to provide saline injections for hydration for the initial 5 days could also have contributed to their stress levels. Another important observation in our study concerning the relationship between neuronal excitability and mechanosensitivity was the lack of correlation between these two factors during the acute infection (i.e. neuronal hyperexcitability but no increase in mechanosensitivity). This finding mirrors a similar observation in studies of mouse small intestinal afferents (Keating et al., 2008) and presumably reflects an altered dynamic at the mucosal – nerve terminal interface related to the net effect of the large repertoire inflammatory mediators or other signalling pathways such as 5-HT release from enterochromaffin cells.

In summary, we found that C. rodentium induced colitis, a model of human E. coli infection, induced hyperexcitability of colonic nociceptive DRG neurons and that these changes in the intrinsic excitability of the neurons persisted after the infection had resolved, due to suppression of I_A and possibly other channel(s). This persistent excitability could contribute to exaggerated
nociceptive signaling in response to a given colonic stimulus and suggests that acute bacterial colitis could alter peripheral mechanisms of pain signaling, thereby contributing to the pain of PI-IBS. Epidemiological studies suggest that psychological factors and female sex may also be contributing factors, demonstrating the potential for interaction between central and peripheral mechanisms (Marshall et al., 2006). Further studies are needed in this model to evaluate these interactions.
CHAPTER 3: MEDIATORS OF BRAIN-GUT INTERACTIONS INCREASE PERIPHERAL NOCICEPTIVE SIGNALING IN A POST-INFECTIOUS IBS MODEL
ABSTRACT

BACKGROUND & AIMS: To investigate the peripheral sensory effects of repeated stress in post-infectious IBS, we tested whether stress following self-limiting bacterial colitis increases colonic dorsal root ganglia (DRG) nociceptive signaling. METHODS: C57BL/6 mice were infected with C. rodentium. Stress was induced using a 9 day water avoidance paradigm (day 21-30 post infection). Colonic DRG neuronal excitability was measured using perforated patch clamp techniques, in vitro multi-unit afferent recordings and measurements of visceromotor reflexes. RESULTS: Combined stress and prior infection increased corticosterone and epinephrine levels compared to infected animals but did not alter the resolution of colonic inflammation. These changes were associated with increased neuronal excitability and parallel changes in multi-unit afferent recordings and visceromotor reflex thresholds. Protease activity was increased in C. rodentium colons at day 30. Protease inhibitors markedly reduced the effects of colonic supernatants on neuronal excitability from C. rodentium but not stressed animals. Colonic DRG neurons expressed β2 adrenergic and glucocorticoid receptor transcripts and incubation with stress mediators recapitulated the effects on neuronal excitability seen with chronic stress alone. Levels of PAR2 activation which had no effect on neuronal excitability had marked effects on neurons from chronically stressed animals. CONCLUSION: These studies demonstrate that stress combined with prior acute bacterial colitis results in exaggerated peripheral nociceptive signaling. Proteases and stress
mediators can signal directly to colonic DRG neurons and these pathways could provide important new targets for treatment of post-infectious IBS.
INTRODUCTION

The irritable bowel syndrome (IBS) is defined by symptom criteria (i.e. Rome III criteria: abdominal pain associated with altered bowel pattern) because there are currently no reliable biomarkers and, as a result, they likely define a group of heterogeneous disorders (Clarke et al., 2009; Spiller & Garsed, 2009a). Of these disorders, the post-infectious IBS (PI-IBS) subgroup is one of the best characterized. PI-IBS symptoms are initiated abruptly by an infectious (typically bacterial) self-limiting colitis. Despite the relatively prompt resolution of infection, normal endoscopic appearance of the mucosa following infection, and absence of histopathological findings, symptoms of diarrhea and pain often persist for years after the initial infection (Clarke et al., 2009; Marshall et al., 2006; Spiller, 2008; Spiller & Garsed, 2009a). Remarkably, following a water borne outbreak of bacterial colitis in Walkerton, Ontario, almost 30% of the population developed PI-IBS and overall, PI-IBS represents somewhere between 6-17% of all IBS cases (Clarke et al., 2009; Spiller & Garsed, 2009a; Vanner et al., 1999). The mechanism(s) which predispose some patients who develop an acute infectious colitis to subsequently experience PI-IBS are unclear, although altered immune signaling within the intestine in these patients and psychological factors may contribute (Clarke et al., 2009; Spiller, 2008; Spiller & Garsed, 2009b; Spiller & Garsed, 2009a; Spiller et al., 2000).

Psychological stress can exacerbate pain syndromes, including IBS (Ford et al., 1995; Gwee et al., 1999; Khasar et al., 2008; Posserud et al., 2004), but the
mechanisms involved are poorly understood. Possible mechanisms may include plasticity in the central nervous system and changes in peripheral signaling by nociceptive dorsal root ganglia (DRG) neurons (Bradesi et al., 2005; Ford et al., 1995; van den Wijngaard et al., 2009; Winston et al., 2010). However, several recent studies in animals provide evidence that stress can signal either directly or indirectly to change the properties of DRG neurons, including those innervating the colon (Bradesi et al., 2005; Khasar et al., 2008; van den Wijngaard et al., 2009; Winston et al., 2010). Moreover, the evidence for immune modulation within the colon in patients with IBS, possibly in response to chronic stress, is most compelling for those patients with PI-IBS. The possible contributions of stress to peripheral pain signaling in the setting of a self-limiting bacterial colitis, and the mechanisms involved, have not been studied.

Human *E. coli* self-limiting colitis, a common cause of PI-IBS, can be closely replicated in mice by *C. rodentium* infection (Ibeakanma et al., 2009; Mundy et al., 2005). Our recent studies have shown that this self-limiting colitis induces a post-infectious hyperexcitability of colonic DRG neurons and hyperalgesia, a dominant feature of PI-IBS in humans (Ibeakanma et al., 2009). This finding set the stage to examine whether repeated stress further exacerbates peripheral pain signaling in PI-IBS, and if so, whether increased levels of stress hormones and/or immune mediators signal to DRG neurons, which in turn alter the properties of the neurons.
METHODS

Animals

All experiments were performed according to the guidelines of Queen’s University Animal Care Committee and the Canadian Council of Animal Care. Male C57BL/6 mice, 6-9 weeks old (25-30 g) were obtained from Charles River Laboratories (Montreal, Quebec Canada).

Post-infectious C. rodentium and water avoidance stress (WAS) models

Under light Isoflurane (0.5 ml) induced anaesthesia, mice were gavaged with 0.125 ml of C. rodentium, strain DBS 100 (1.06 x 10^{10} CFU) or saline using a flexible non-toxic polyethylene tube (I.D. 76 mm and O.D. 1.22 mm) as previously described (Ibeakanma et al., 2009; Mundy et al., 2005). Subcutaneous injections of 0.5 ml lactated Ringers solution were administered daily to C. rodentium gavaged mice for the first 7 days to avoid dehydration. Animals were euthanized at day 10 or day 30 (a time point at which infection and inflammation had resolved) for further studies (Ibeakanma et al., 2009).

Repeated stress was induced in either control or C. rodentium infected mice (starting from day 20 post-infection) using a 9 day water avoidance paradigm. Mice were placed on a dry platform (40 mm diameter) in a bucket of water (280 mm diameter) for 1 hour each day, standing 10 mm above water level and 230 mm below the brim of the bucket. Control mice were handled, but were not exposed to the stress.
**Behavioral assessment**

An ENV-560A elevated plus maze for mice (Med Associates Inc., St. Albans, Vermont, USA) was used to assess anxiety-related behavior (Rodgers & Dalvi, 1997). Control or repeated water avoidance stress (WAS) mice were acclimatized to the test room under low intensity red light for 60 minutes on the 2 days preceding plus maze test, at the same times of day to be used for the test. Plus maze exploration occurred 2 hours after the final WAS session on test day. Each mouse was placed at the intersection of the arms, and filmed with a mounted digital camcorder during 5 minutes of exploration. Control and WAS mice were interleaved to prevent time-related effects upon the data. The maze was cleaned with 70% ethanol and dried before and after each mouse was tested. Entrances to, and time spent in open and closed arms were recorded from when all 4 paws crossed into the arm. A partial exit (1-3 paws out) before re-entry to an arm was subtracted from the time spent in the arm, but did not count as a second arm entry.

**Histopathology assessment**

Colons from post-infected *C. rodentium* or combined WAS and *C. rodentium* infected animals were fixed in 4% paraformaldehyde and embedded in paraffin for histopathological assessment. 5 μm thick sections were cut and stained with hematoxylin and eosin (H&E). Slides were reviewed and assessed for colon pathology by a pathologist blinded to the treatment groups utilizing a
semi-quantitative colitis histological scoring system that evaluated and scored for inflammation, crypt height, recent and chronic damage, as described previously (Ibeakanma et al., 2009).

**Enzyme linked immunosorbent assay (ELISA)**

Within 1-2 hrs following the last WAS session, mice were anesthetized and blood drawn by intra-cardiac puncture for assay of epinephrine and corticosterone levels using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA). Assay procedures were performed following protocols provided in kits by the manufacturer. Assay plates were placed in automated microplate reader (Titertek Instruments, Huntsville, Alabama, USA), and sample absorbance at 450 nm (wavelength correction set at 595 nm) measured.

**Supernatants from colonic tissue**

Under isoflurane-induced anesthesia, the thoracic and abdominal cavities of control, WAS or post-infected C. rodentium mice were opened and animals euthanized by trans-cardiac perfusion. The distal colon was excised in one piece, flushed with normal saline (to remove fecal materials) and weighed. The colon was cut into ~ 2 mm segments and each incubated overnight with 250 µl RPMI media, with or without a global protease inhibitor (Sigma-Aldrich, Oakville, ON, Canada), in an incubator at 37 °C, 95% O₂ and 5% CO₂. After 24 h incubation, the supernatants were collected and stored at -80 °C for further use.
Fast-Blue surgeries for labeling of colon projecting DRG neurons

To identify DRG neurons projecting to the colon, surgeries were performed on control, WAS or post-infected C. rodentium animals at day 3 or 20 post-infection and the retrograde tracer Fast-Blue (Cedarlane Laboratories; Hornby, ON, Canada) injected into the wall of the colon, as described (Ibeakanma et al., 2009). Mice were anesthetized with ketamine-xylazine (0.015 mg - 0.001 mg/g of weight) and subjected to mid-line laparatomy, the colon was carefully exposed and the retrograde marker Fast Blue (1.7% wt/vol in sterile water) injected in small volumes (1-2 µl) into multiple sites on the colon wall. The gut was wiped down after each injection to remove excess dye and to avoid accidental labeling of unwanted tissues. The colon was placed back into the abdominal cavity and the incision sutured. Mice were kept on a warm bed until fully recovered and, were allowed to feed and drink ad libitum.

Enzymatic dissociation of DRGs

Mice were euthanized following intra-peritoneal injection of combined ketamine – xylazine (0.02mg/ml – 0.002mg/ml) and DRGs from thoracic vertebra T_9 to T_13 isolated bilaterally as previously described (Ibeakanma et al., 2009). Briefly, isolated ganglia were dissociated into single cell bodies using a two-step enzymatic digestion process. The ganglia were first incubated for 10 min at 37 °C in papain solution (69 U papain activated with 1 mg L-cysteine and 3 µl NaHCO_3 / 1.5 ml HBSS), and then for another 10 min in collagenase – dispase solution (12
mg collagenase plus 14 mg dispase / 3 ml HBSS). Following incubation in enzymes, DRGs were washed once with 2 ml warm F12 medium containing 10% FCS, and subsequently centrifuged for 1 min at < 800 rpm. After careful supernatant removal, ~ 500 μl F12 medium was added to the mildly digested DRG. Trituration of ganglia was gently performed ~ 10 times using a glass flame-polished Pasteur pipette. Neurons were then plated onto cover slips pre-coated with sterile laminin / poly-D lysine (0.017 mg/ml and 2 mg/ml) in 12-well culture plates. Cells were cultured in F12 medium, containing 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) and maintained at 37 °C in humidified atmosphere of 5% CO₂.

**Patch clamp recordings from dorsal root ganglia (DRGs) neurons**

Following overnight culture, perforated patch clamp recordings in current clamp were obtained. Nociceptive neurons were identified by their small size (≤ 40 pF) and colon specific neurons by the Fast-Blue fluorescence emitted from labeled cell-bodies under short exposure to ultra violet light.

Membrane electrical properties were recorded from cultured DRG neurons using a Multiclamp 700B or Axopatch 200B amplifier connected to Digidata 1440A and 1322A AD interfaces (Molecular Devices, Sunnyvale, CA, USA.) respectively. Acquired data were stored in a PC, processed using pClamp 10.1 software (Molecular Devices, Sunnyvale, CA, U.S.A.), and analyzed with GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, CA, USA). Patch pipettes
made of thin-wall glass capillary tubes with no internal filament (Warner Instruments) were employed. The pipettes were pulled using a Narishige PP-830 puller, and polished with a Narishige MF-830 micro forge to a resistance of between 2-5 MΩ when immersed in the bath solution. The recording chamber was continuously perfused with external solution at an approximate rate of 2 ml/min. Overnight primary cultures were utilized to prevent loss of Fast-Blue fluorescence, minimize toxicity and to avoid space-clamp and size measurement problems that may result from neurite overgrowth. Experiments were performed at room temperature (~23 °C). Changes in excitability of the neurons were assessed by measuring rheobase and numbers of action potentials discharged at twice the rheobase, as described (Ibeakanma et al., 2009). To ensure adequate numbers of neurons for patch clamp recordings, only two groups of animals could be studied at any one time.

Overnight incubation studies with protease-activated receptor 2 activating peptide (PAR2-AP) were performed on neurons isolated from mice exposed to WAS or controls. In some experiments, neurons were incubated in supernatants obtained from colonic tissues. In other experiments, neurons were incubated overnight in supernatants which also contained a global protease inhibitor (Sigma-Aldrich, Oakville, ON, Canada).

Standard solutions used had the following compositions (in mM): Pipette solution - K-Gluconate 110, KCl 30, HEPES 10, MgCl₂ 1, and CaCl₂ 2; pH adjusted to 7.25 with 1M KOH. External solution - NaCl 100, KCl 5, HEPES 10,
glucose 10, MgCl$_2$ 1, and CaCl$_2$ 2; pH adjusted to 7.3-7.4 with 3M NaOH. To establish electrical connection between the recording electrode and intracellular space, Amphotericin B (240 µg/ml) was added to the internal solution to perforate the cell membrane.

**In vitro multi-unit afferent recordings**

At day 30 post *C. rodentium* infection, infected and stressed mice were euthanized, as described above, and the distal colon and rectum were dissected out with attached mesenteric nerves. Proximal and distal ends of the isolated bowel with attached mesenteric nerves were securely attached to an input and outlet port. The input port was connected to a perfusion pump, which allowed continuous intra-luminal perfusion of oxygenated Krebs solution (at 33 - 34 °C) through the segment (0.2 ml/min) or periodic distension when closed. Intra-luminal pressure was recorded via a pressure amplifier (NL 108, Digitimer, UK) connected in parallel with the input port. The mesenteric bundle was pinned out to the base of the recording chamber and a mesenteric nerve was dissected out from the bundle and drawn into a suction electrode. The electrical activity was recorded by a Neurolog headstage (NL 100, Digitimer Ltd, UK), amplified (NL104), filtered (NL125 band pass 200-3000 Hz) and acquired (20 kHz sampling rate) through a Micro 1401 MKII interface running Spike 2 software (Cambridge Electronic Design, UK) to a PC. The preparation was stabilized for 30 minutes and then distended to an intra-luminal pressure of 60 mmHg with
closure of the outlet port. Multi-unit afferent discharge responses to ramp distensions were recorded, as described (Ibeakanma et al., 2009). Only afferent discharge responses to ramp distensions that were stable and reproducible (at least 3 times 10 min apart) were analyzed. In each case, only the third response was analyzed.

**Visceromotor reflexes**

Visceromotor responses to colorectal distensions were recorded, as previously described (Cenac et al., 2007). Mice were anaesthetized by xylazine, ketamine, and saline (1:20:29 ratio, intraperitoneally). Two electrodes (Bioflex insulated wire AS631; Cooner Wire) were implanted in the abdominal external oblique musculature. Electrodes were exteriorized at the back of the neck and protected by a plastic tube attached to the skin. Three days after electrode implantation, colorectal distension was performed on saline, *C. rodentium*, stress and *C. rodentium* plus stress treated animals. Mice were placed in plastic tunnels (3-cm diameter, 10-cm length) in which they could not move, escape, or turn around. The wires were connected to a Bio Amp (ADInstruments) itself connected to a PowerLab (ADInstruments) used as an electromyogram acquisition system using Chart 5 software (ADInstruments). Colorectal distension was then performed in a stepwise fashion. The balloon (10.5-mm diameter) used for distensions was an arterial embolectomy probe (Fogarty Thru-Lumen Catheter 4F; Edwards Lifesciences, Maurepas, France). Rectal distension was
performed by insertion of the balloon into the colon at 5 mm proximal to the rectum. The balloon was then inflated in a stepwise fashion from 0 to 60 mmHg in 15-mmHg increments. Ten-second distensions were performed in triplicate at pressures of 15, 30, 45, and 60 mmHg with 5-min intervals.

**RT-PCR**

Laser captured Fast-Blue labeled DRG neurons were analyzed for the expression of α-GCR and β₂-AR following standard semi-quantitative RT-PCR protocols. Total mRNA was obtained from neurons using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. First strand Oligo(dT)-primed cDNAs were synthesized from 0.5 μg total RNA using Oligo dT and SuperScript III (Invitrogen, Carlsbad, CA, USA) for 50 min at 50 °C. The resultant cDNAs (0.5 μl) were used as a template for PCR amplification in a 25 μl reaction volume, containing 2.5 μl buffer 10x, 200 nM dNTP's, 1.5 mM MgCl₂, 0.2 μM of each sense and antisense primers and 0.1 μl taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The following reaction conditions were used for amplification of each cDNA in the linear range: α-GCR, 38 cycles of 94 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 1 min; β₂-AR, 38 cycles, 94 °C for 30 sec, 61 °C for 30 sec and 72 °C for 1 min; and β-actin, 35 cycles, 94 °C for 30 sec, 61.5 °C for 30 sec and 72 °C for 1 min. A final extension period of 8 min at 72 °C was allowed for all cycles. No-template negative control reactions were performed to rule out genomic DNA contamination of the template RNA.
Primers were designed and ordered from Invitrogen (Carlsbad, CA, USA). See table 1 for primer sequences, GenBank accession numbers and sizes of the PCR products. The PCR products (DNA samples) were loaded onto agarose gels (2 %, w/v) containing ethidium bromide (10 μg/ml) and results photographed.

### Table 3-1 Primers used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size (bp)</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-GCR</td>
<td>355</td>
<td>ATC CTT AGC TCC CCC TGG TA</td>
<td>CTG TGG GTA GCC CAA GTC AT</td>
</tr>
<tr>
<td>β2-AR</td>
<td>386</td>
<td>ACC AAG AAT AAG GCC CGA GT</td>
<td>GTC TTG AGG GCT TTG TGC TC</td>
</tr>
</tbody>
</table>

GenBank accession no: DQ504162 (α-GCR); NM007420 (β2-AR)

### Western blot analysis of occludin

Western blot experiments were performed with total protein extracts to examine expression of the tight junction protein, occludin, in WAS or day 30 C. rodentium, combined WAS and C. rodentium, or saline gavaged colonic tissues. Mice were anesthetised with isoflurane (0.5 ml) and perfused with 30 ml of chilled HBSS by trans-cardiac perfusion to minimize blood contamination of tissue. Colons were dissected, cleaned of feces, flash frozen in liquid N₂ and stored at -80 °C. Frozen colons were powdered under liquid N₂ by mortar and pestle. Powdered tissues were homogenized in SDS Lysis buffer containing 1% w/v SDS, 100 mM Tris HCl pH 6.8, 10% glycerol, 1x protease inhibitor cocktail (Sigma-Aldrich, Oakville, ON, Canada) and 1x phosphatase inhibitor cocktail (Sigma-Aldrich, Oakville, ON, Canada). Insoluble material was pelleted by
centrifugation at 21,000 x g for 20 min, and supernatants (soluble total protein extract) collected and stored at -80 °C until when needed. Micro BCA Protein Assays Kits (Thermo Scientific, Rockford, USA) were used to quantify the protein extracts. Total protein extracts (50 μg) were separated according to size on 12% acrylamide SDS-PAGE gels (w/v) and transferred to nitrocellulose membrane in chilled Tris-Glycine transfer buffer containing 25 mM Tris, 192 mM Glycine, 10% v/v Methanol, 0.1% SDS at 400 mA for 2 hrs. Pre-stained standard molecular weight proteins were used as reference. Membranes were blocked with Western Blocker Solution (Sigma-Aldrich, Oakville, ON, Canada), and then probed with anti-occludin primary antibody (1:500 dilutions). Following secondary antibody-HRP incubation and membrane exposure to chemiluminescent substrate, membranes were exposed to x-ray film and developed. Films were scanned and digitized for analysis.

**Assay of Trypsin-like activity in colonic tissues**

Trypsin-like activity in post-infected *C. rodentium* or saline gavaged groups was measured as previously described (Hansen *et al.*, 2005). Mice were euthanized and the entire colon dissected out and flushed twice with 1 mL PBS to remove feces. Trypsin-like activity was measured in ~ 1 cm cut segments of the distal colon using tosyl-Gly-Pro-Arg-p-nitroanilide (150 μM; Sigma, Saint Quentin Fallavier, France) as substrates. 20 μL of samples were re-suspended in buffer containing 100 mM Tris/HCl and 1 mM CaCl₂; pH 8. Optical densities of
plates were measured at 405 nm over 30 minutes at 37 °C with a microplate reader (NOVOstar; BMG Labtech, Champigny, France) and compared with those of known standard dilutions of trypsin from porcine pancreas (Sigma). Protein concentration was determined using a BCA kit (Pierce, Thermo Scientific, Courtaboeuf, France) and results expressed as U trypsin/mg protein.

**Measurement of protease activity**

Protease activities in colonic tissues were determined using activity-based probes, small molecule inhibitor-based probes that covalently interact with only activated proteases. These probes are comprised of an inhibitor group (warhead) that provides specificity, a linker to prevent steric congestion and to provide further selectivity, and a tag for detection. To detect serine proteases, a probe was used with a diphenylphosphonate warhead and a biotin tag for detection: biotin-Pro-Lys-diphenylphosphonate (Bio-PK-DPP). To detect cysteine proteases, a probe was used with an acyloxymethylketone warhead and a near infrared Dye 800CW for detection: GB138. Colonic samples were homogenized and sonicated in HBSS, HEPES 10 mM, pH 7.4, and centrifuged. Supernatants (40 µg of protein) were incubated with Bio-PK-DPP or GB138 (1 µM probe, 1 h, room temperature). Bio-PK-DPP-bound proteases were detected by Western blotting for biotin using mouse anti-biotin (1:5000, Jackson) and goat anti-mouse coupled to AlexaFluor® 680 (1:10000, Molecular Probes). GB138-bound proteases were detected by in-gel fluorescence (IR800 channel). Results were
normalized to β-actin, detected by Western blotting using a mouse anti-β-actin (1:10000, Sigma) and a goat anti-mouse coupled to AlexaFluor® 680 or IRDye™800. Blots and gels were analyzed with Odyssey infrared imaging system (LiCor, Lincoln, NE).

Data analysis

Data are expressed as the mean ± SEM. Statistical analysis was performed using student t-test or 2-way ANOVA with Bonferroni post-tests, significance was p< 0.05.

Substances

The activity based probe Biotin-Pro-Lys-Diphenylphosphonate (Bio-PK-DPP), which covalently interacts with serine proteases, was from Dr. M. Hollenberg (University of Calgary). GB138, which interacts with cysteine proteases, was from Dr. M. Bogyo (Stanford University). Amphotericin B (Sigma-Aldrich, Oakville, ON, Canada) stock solution (60 µg/µl DMSO) was made fresh daily. HBSS and F12 medium were purchased from GIBCO (Invitrogen Corporation. Van Allen Way Carlsbad, CA, USA). PAR2-AP was purchased from Peptides International (Louisville, Kentucky, USA). Poly-D lysine was purchased from VWR (West Chester, Pennsylvania, USA). Papain was purchased from Worthington (Lakewood, NJ, USA). All other substances were purchased from Sigma-Aldrich (Oakville, ON, Canada).
RESULTS

**WAS induces HPA-SA axis activation and anxiety related behaviors**

Serum epinephrine levels were almost 5-fold higher (n = 8 animals in each group, p = 0.022) and corticosterone levels were more than 2-fold higher (n = 3 animals in each group, p = 0.016) in WAS mice compared to controls (Figure 3-1A and B). WAS animals also displayed less open arm time in elevated plus maze performance testing (Figure 3-1C), suggesting the stressed animals exhibited anxiety-like behavior.

WAS had no effect on measurements of colonic inflammation and did not affect protein expression of the zona occludens -1 tight junction protein (see supplementary figures 3-1 and 3-2 [Appendix]).

**WAS induces neuronal excitability in colonic DRG neurons**

To examine the effects of WAS alone on DRG neuron excitability, patch clamp recordings were obtained from Fast-Blue labeled neurons following 9 days of stress (Figure 3-2A.). Action potential discharge at twice the rheobase increased by 80% in WAS group (4.16 ± 0.51, n = 18 neurons), compared to control group (2.30 ± 0.47, n = 13 neurons; P = 0.0167). No differences were observed in rheobase, membrane potential and input resistance.
Figure 3-1 Chronic stress (WAS) increases HPA-SA axis activation. (A) Time line of WAS. (B) Bar graph of serum epinephrine (left) and corticosterone (right) levels from non-stressed (control) or stressed (WAS) animals. (C) Schematic illustration of the apparatus (plus-maze) used for assessment of anxiety-related behavior in WAS animals (left) and a summary graph showing the percentage of time spent in the open arm by control or WAS animals (right). Numbers in parenthesis represent number of animals from each group. Data are reported as mean ± SEM, *p < 0.05.
Figure 3-2 Chronic stress (WAS) increases excitability of colonic DRG neurons. (A) Representative trace of action potentials evoked by a 500 ms depolarizing pulse at rheobase (left panel) and twice the rheobase (right panel) from neurons isolated from stressed (upper traces) and non-stressed (lower traces) animals. (B) Bar graphs summarizing changes in the electrical properties of colonic sensory neurons brought about by 9 days paradigm of (WAS) stress. Neurons from stressed animals fired significantly more action potentials than controls. Numbers in parenthesis represent number of neurons from each group. Data are reported as mean ± SEM, *p < 0.05.
Repeated stress combined with post *C. rodentium* infection increases HPA-SA activation and exaggerates peripheral post-infectious sensory signaling in DRG neurons

As shown in the schematic study paradigm in figure 3-3, mice were subjected to WAS from day 21 following *C. rodentium* infection and serum samples obtained at day 30 for ELISA measurement of corticosterone and epinephrine. Corticosterone levels were two-fold higher (n = 7 animals in each group, p = 0.01) and epinephrine levels were almost 3 times higher (n = 6 animals in each group, p = 0.033) in animals previously exposed to *C. rodentium* infection plus WAS compared to infected mice alone.

We have previously shown that action potential discharge at twice the rheobase is significantly increased in colonic DRG neurons from day 30 post-infected *C. rodentium* animals (Ibeakanma *et al.*, 2009) compared to controls. We now examined the added effect of stress by first performing patch clamp studies comparing neuronal excitability in neurons isolated from the WAS and non-WAS day 30 post-infected *C. rodentium* animals. As shown in figure 3-3C, a combination of WAS and *C. rodentium* infection produced greater effects on excitability of the neurons. Compared to the *C. rodentium* treated neurons alone (n = 10 neurons), *C. rodentium* infection plus WAS (n = 12 neurons) significantly decreased the rheobase by 38% (mean = 55.83 ± 7.63 pA vs. 89.00 ± 13.94 pA for *C. rodentium* plus stress and *C. rodentium* alone, respectively; p = 0.041) and increased the action potential firing at twice the rheobase by 70% (mean = 4.08 ±
0.46 vs. 2.40 ± 0.63 for *C. rodentium* plus stress and *C. rodentium* alone, respectively; *p* = 0.041). No differences were observed in the membrane potential and input resistance (data not shown).

**WAS combined with post *C. rodentium* infection induces visceral hyperalgesia and allodynia**

To further characterize changes in peripheral sensory signaling evoked by the WAS and post *C. rodentium* infection, we measured changes in multi-unit afferent discharge and visceromotor responses following colorectal distention (CRD). In *in vitro* multi-unit afferent recordings, nerve discharges evoked by CRD were significantly increased in colons from animals previously infected with *C. rodentium* and subjected to repeated stress compared to the *C. rodentium* infected animals alone. (Figure 3-4, *p* < 0.001 two-way ANOVA; *n* = 4 colons per group). There were no differences (unpaired t-test, *p* > 0.05) in spontaneous activity (*C. rodentium* = 44.6 ± 6.4 spikes/s vs. *C. rodentium* plus WAS 30.5 ± 7.3 spikes/s). Our previous studies demonstrated that nerve discharges from colons of *C. rodentium* alone were significantly increased compared to controls (Ibeakanma et al., 2009).

Visceromotor responses to colorectal distention (CRD) were measured in four groups; saline controls, post *C. rodentium* infection, WAS, and post *C. rodentium* infection and WAS. The chronic stress and the combined post *C. rodentium* infection and chronic stress groups exhibited hyperalgesia, with
Figure 3-3 Chronic stress (WAS) combined with prior *C. rodentium* infection-augmented HPA-SA axis activation and increased excitability of colonic DRG neurons. (A) Schematic drawing showing the time line of the WAS and *C. rodentium* infection and ELISA measurements of epinephrine and corticosterone and electrophysiological recordings (day 30). (B) Bar graph summarizing serum levels of corticosterone (left) and epinephrine (right) in *C. rodentium* alone and *C. rodentium* plus stress animals. (C) Summary data illustrating the combined effects of post *C. rodentium* infection and stress on excitability of colonic DRG neurons. Stress plus post *C. rodentium* infection not only significantly increased action potential discharges at twice the rheobase but also decreased the rheobase. Numbers in parenthesis represent number of animals from each group. Data are reported as mean ± SEM, *p < 0.05.*
Figure 3-4 Combined post C. rodentium infection plus chronic stress (WAS) augmented colonic afferent mechanosensitivity and induced visceral hyperalgesia and allodynia in mice. (A) Representative trace of multi-unit afferent nerve discharges recorded from colons of day 30 post-infected C. rodentium or C. rodentium plus WAS animals in response to ramp distension. Top traces: ramp distension; Middle traces: nerve activity histogram; Bottom traces: raw recording of whole nerve activity. (B) Summary of distention-response profiles of multi-unit afferents recorded from colons of C. rodentium (solid circles) or C. rodentium plus WAS (open circles) animals. (C) Summary data (n = 7-8 animals per group) illustrating VMR responses to graded CRD (15, 30, 45 and 60 mmHg pressure) in mice subjected to saline-only (solid square), C. rodentium (solid circle), WAS (solid triangle) or C. rodentium plus WAS (open circle) treatment. Visceral hyperalgesia was induced in WAS plus C. rodentium and WAS alone groups. Only the C. rodentium plus WAS group exhibited evidence of allodynia (dotted box). Data are reported as mean ± SEM, ***p < 0.001 (two-way ANOVA with Bonferroni correction).
significant increases in visceromotor responses following 30, 45 and 60 mmHg CRD (Figure 3-4; p<0.001, n= 7-8 animals per group). Only the combined post C. *rodentium* and chronic stress group exhibited evidence of allodynia compared to all other groups.

**Proteases in post C. *rodentium* increase the excitability of the colonic nociceptive DRG neurons**

To understand the mechanisms underlying the post C. *rodentium* and WAS effects on peripheral sensory signaling we examined colonic tissue protease actions, given the growing evidence that protease signaling is abnormal in IBS patients (Barbara *et al.*, 2004b; Cenac *et al.*, 2007). We found that protease-like activity was significantly elevated in post C. *rodentium* colonic tissues compared to controls (; n = 10 specimens for each group, p < 0.01) and that levels of serine proteases were increased (43%; p<0.01), when measured using activity based probes. Levels of cysteine proteases, however, did not differ between the groups (Figure 3-5 and supplementary figure 3 [Appendix]).

We next examined the actions of tissue proteases on neuronal excitability by incubating neurons in supernatants obtained from colonic tissue in the presence or absence of a global protease inhibitor (Figure 3-5). When supernatants from colons of post C. *rodentium* animals were examined in the presence of the protease inhibitor, the rheobase was almost 43% higher (p = 0.016) and the action potential number at twice the rheobase 69% lower (p =
0.007). This suggests that tissue proteases in the colons played a significant role in the hyperexcitability observed in post C. rodentium animals. To determine whether chronic stress altered protease signaling as well, we conducted similar experiments using colons from stressed mice. The protease inhibitor had no effect on the level of neuronal excitability in neurons incubated in supernatants from these animals (Figure 3-5C). Based on these findings, the actions of repeated stress on nociceptive signaling do not appear to be mediated by changes in tissues proteases, unlike the effects in the post C. rodentium infected animals.

**Stress mediators epinephrine and corticosterone directly modulate colonic DRG neurons**

Previous studies in the somatic nervous system suggest that the stress hormones epinephrine and corticosterone induce hyperalgesia following chronic stress by signaling directly to DRG neurons and altering their intrinsic properties (Khasar et al., 2008). We therefore examined whether similar pathways could exist in the colonic viscera. We first sought evidence that the α-glucocorticoid- (α-GCR) and β₂-adrenergic (β₂-AR) receptors are expressed on colonic DRG neurons. Fast-Blue labeled DRG were laser captured and mRNA transcript identified by RT-PCR (Figure 3-6).

To determine whether these stress hormones could recapitulate the effects of WAS on DRG neuronal excitability (see Figure 3-2), we incubated DRG
Protease signaling is important in *C. rodentium* induced excitation of colon sensory neurons. (A) Summary graphs illustrating trypsin-like- (left), serine protease (center) - and cysteine protease (right) - activities in mouse distal colonic tissues at day 30 post-infection. Trypsin-like and serine protease activities, but not cysteine proteases, are elevated following *C. rodentium* infection compared to water gavage controls. 10 colons were analyzed in each group. (B) Bar graph illustrating decreased excitability (increased rheobase and decreased action potential firing at twice the rheobase) of neurons incubated in colonic supernatants from *C. rodentium* infection plus a non-selective protease inhibitor compared to those incubated in supernatants without the inhibitor. (C) Summary data demonstrating lack effect of the non-selective protease inhibitor with colonic supernatants from WAS animals. Numbers in parenthesis represent number of impaled neurons from each group. Data are reported as mean ± SEM, *p < 0.05, **p < 0.01, student t-test or one-way ANOVA.
Figure 3-6 Stress mediators can signal directly to colonic DRG neurons. (A) RT-PCR products demonstrating expression of glucocorticoid- (middle gel; 355 bp product) and β2-adrenergic (right gel; 386 bp product) mRNA transcript in colonic DRG neurons. Fast blue labeled colonic neurons were obtained using laser capture microdissection. (B) Bar graphs summarizing evidence of direct activation of colonic sensory neurons by stress mediators, corticosterone and epinephrine. Overnight incubation of colon sensory DRG neurons with 1 µM corticosterone and 5 nM epinephrine significantly decreased the rheobase and increased action potential discharges at twice the rheobase compared to control neurons. (C) Colonic DRG neurons were incubated overnight in colonic supernatants from control or WAS animals. There was no difference in the level of neuronal excitability between the two groups, suggesting WAS does not significantly increase the net signaling of pro-nociceptive tissue cytokines. Numbers in parenthesis represents number of impaled neurons from each group. Data are reported as mean ± SEM, *p < 0.05.
neurons overnight with epinephrine (5 ng) and corticosterone (1 µM). Compared to control neurons, the rheobase decreased by 56% (p = 0.031) and action potential discharges at twice the rheobase increased by more than 60% in treated (p = 0.040) compared to non-treated cells. We also tested if the excitability of neurons incubated in supernatants from control mice differed from neurons incubated with supernatants from colons of WAS mice. No differences were seen in the level of neuronal excitability between these two groups (Figure 3-6C). Together, these data support the contention that stress mediators released following WAS can signal directly to sensory DRG neurons to alter their electrical properties.

**WAS sensitizes colonic DRG neurons to sub-threshold protease signaling**

We explored the potential interaction of protease and stress mediator signaling by examining if exposure to chronic stress could sensitize neurons to sub-threshold levels of protease signaling. To do so, we conducted preliminary studies using patch clamp recordings from Fast-Blue labeled DRG neurons from control animals and established that incubation of the neurons with the protease-activated receptor 2 activating peptide SLIGRL (PAR2-AP; 30 µM) had no effect on excitability (data not shown). Patch clamp recordings were then obtained from four groups of neurons; controls, controls incubated in PAR2-AP 30 µM, WAS, and WAS incubated in PAR2-AP 30 µM (Figure 3-7). These studies showed that the PAR2-AP incubated with the control neurons had no effect but
Figure 3-7 Stress mediators and protease activation had a synergistic effect on colonic DRG neuronal excitability. Data summarizing rheobase and action potential numbers at twice the rheobase of neurons from control or WAS neurons exposed to sub-threshold levels of protease activating peptide 2 (PAR2-AP; 30 µM). PAR2-AP alone had no effect on rheobase or action potential discharge number but when combined with neurons from WAS animals, PAR2-AP induced neuronal excitability indicated by the significantly decreased rheobase (left graph). Data are reported as mean ± SEM, **p < 0.01.
when incubated with neurons from animals exposed to WAS, there was a profound decrease in the rheobase (79%; p< 0.002). These data suggest that chronic stress mediators can sensitize colonic DRG neurons to sub-threshold levels of inflammatory mediators such as proteases.

DISCUSSION

This study examined peripheral nociceptive mechanisms which underlie the strong association between psychological stress and the development of PI-IBS (Gwee et al., 1999; Marshall et al., 2006). We tested whether abnormal nociceptive signaling in peripheral DRG neurons results from a “brain - gut” interaction using a well recognized model of human E. coli infection, the C. rodentium mouse model of self-limiting colitis. The key finding was that visceral hyperalgesia and allodynia were significantly increased when animals recovering from the bacterial infection were subjected to repeated stress. The implication is that patients experiencing psychological stress following their infection will likely suffer more pain, the most debilitating of the IBS symptoms (Cain et al., 2006; Spiller & Garsed, 2009a) and, as a result, are more likely to express PI-IBS and seek the help of physicians.

There has been considerable debate concerning the relative role of central (CNS) versus peripheral sensory mechanisms in IBS. Peripheral mechanisms may be particularly important in PI-IBS however, given that infection is the triggering event and the evidence for subsequent altered immune signaling in the
colon (Lee et al., 2008; Spiller et al., 2000), including elevated tissue proteases (Amadesi & Bunnett, 2004; Cenac et al., 2007). We (Figure 3-2) and others have shown repeated stress alone or acute self limiting colitis (Ibeakanma et al., 2009; Khasar et al., 2008) can result in increased excitability of colonic nociceptive neurons. We now show that when combined with the effects of an acute bacterial infection, the nociceptive signaling is greatly magnified, providing further evidence for the importance of peripheral nociceptive mechanisms in PI-IBS. It is also possible that these actions may exacerbate one or more central pathways known to be important in IBS. For example, there is evidence of altered sensory processing of peripheral signals and increased expression of anxiety and hypervigilance of gut symptoms (Anand et al., 2007). Thus, exaggerated peripheral signaling may also exacerbate this abnormal central sensory processing and in turn, further drive the release of stress hormones through repeated activation of the HPA-SA axis. This positive feedback loop could ultimately play a role in the generation of chronic symptoms by recurrent or sustained sensitization of peripheral sensory signaling from the colon.

The finding that protease levels were increased in colonic tissues from our post-infected animal model parallels observations in many human studies of IBS, including PI-IBS (Cenac et al., 2007; Gecse et al., 2008). Proteases are released by mast cells, enterocytes, and activated immune cells and can signal to colonic DRG neurons by cleaving protease activated receptors (PARs), a family of 4 G-protein coupled receptors expressed on DRG neurons (Amadesi & Bunnett,
but also many other cellular targets in the colon (Amadesi & Bunnett, 2004; Bohm et al., 1996). To further test whether the elevated proteases affect neuronal excitability by directly signaling to the neurons, we examined the effects of non-selective protease inhibitors on the actions of supernatants from colonic tissues of post-infected animals. These in vitro patch clamp studies suggested that proteases were important mediators underlying neuronal excitability and that direct signaling to the neurons was a key pathway. We also found evidence that serine proteases may be involved, using activity based probes. Further studies are needed to clarify the relative role of protease subtypes and the intracellular signaling pathways involved.

Previous studies suggest that stress mediators, such as corticosterone and epinephrine, act by signaling directly to adrenergic and corticosterone receptors on DRG neurons, or alternatively, by increasing levels of other pro-nociceptive mediators following immune activation in the colon (Khasar et al., 2008; van den Wijngaard et al., 2009; Winston et al., 2010). For example, whole animal studies examining chronic stress in the somatic nervous system demonstrated that these mediators can signal directly to DRG neurons (Khasar et al., 2008). Alternatively, the repeated stress has been shown to alter intestinal permeability and immune signaling in the colon in other models (Santos et al., 2001; Winston et al., 2010). We found that epinephrine and corticosterone serum levels were elevated following repeated stress in our model and tested whether they might signal to the colon to alter immune signaling using several strategies.
Firstly, we examined the histopathology of the tissues to determine whether differences in the levels of inflammatory markers existed and failed to find any meaningful differences. In parallel, we also examined expression of the tight junction protein ZO-1, given reports that some stress models inhibit ZO-1 levels, resulting in increased mucosal permeability (Barreau et al., 2008; Vicario et al., 2010), but we did not detect any changes in our model. To complement these anatomical studies, we studied the effects of supernatants from the colons of stressed animals to determine if they caused increased excitability of the neurons, as observed in the neurons from the stressed animals. These studies showed no effect compared to controls and, unlike the supernatants from the colons of C. rodentium mice, protease inhibitors had no effect. Together this suggested that altered immune signaling did not play a major role and we therefore tested whether these stress mediators could act by signaling directly to the neurons. We found evidence that colonic DRG neurons express corticosterone and β2-adrenergic receptors and showed that incubating colonic DRG neurons in physiological concentrations of these mediators (Figure. 3-4) caused a similar increase in the excitability of the neurons to that seen following repeated stress.

We also hypothesized that the two signaling pathways identified in this study, i.e. stress mediators and protease signaling to the neurons, might act synergistically, and if so, unmask sub-threshold peripheral signals. We tested this potential by examining sub-threshold levels of protease signaling on DRG
neuronal excitability and found that following repeated stress, this signaling was markedly enhanced (Figure 3-7). Thus, repeated stress may also sustain PI-IBS symptoms by enabling peripheral signaling through pathways that otherwise would not be detectable. These synergistic actions could result from changes at the receptors level e.g. increased GPCR expression, altered second messenger signaling and/or changes in downstream ion channels that underlie action potential electrogenesis such as \( \text{Na}_v \) 1.8 channels or voltage gated \( K^+ \) channels.

**SUMMARY**

This study examined brain-gut interactions observed in PI-IBS patients. We found that repeated stress activates the HPA-SA axis resulting in increased levels of epinephrine and corticosterone and when this stress is combined with prior bacterial self-limiting colitis, neuronal excitability and signs of visceral hyperalgesia/allodynia were markedly exaggerated. The findings highlight the potential role of peripheral nociceptive signaling in PI-IBS and provide a number of novel targets for the treatment of this disorder.
CHAPTER 4: LYSOSOMAL CYSTEINE PROTEASES CONTRIBUTE TO VISCERAL HYPERSENSITIVITY IN PATIENTS WITH POST-INFECTIOUS IRRITABLE BOWEL SYNDROME (PI-IBS)
ABSTRACT

BACKGROUND & AIMS: The poor understanding of the mechanisms responsible for pain expression in PI-IBS makes effective control of this symptom difficult. We sought to understand whether secreted cysteine proteases are important factors contributing to pain in these patients, using the post-infected C. rodentium animal model of PI-IBS and human IBS patient colonic supernatants.

METHODS: Mice were gavaged with C. rodentium or saline. At day 30, colonic tissues were processed to produce mouse colonic supernatants. Human supernatants were obtained from colon biopsies taken from IBS patients or healthy controls undergoing colon screening. Perforated patch clamp recordings were performed on Fast Blue-labeled colonic DRG neurons incubated with cathepsin-s (Cat-S), trypsin, post-infected mouse colonic- or IBS- supernatants in the presence or absence of a cysteine protease inhibitor, E-64. Visceromotor responses (VMR) to colorectal distention (CRD) were employed to assess effects of cysteine proteases on visceral hypersensitivity in mice that received an intra-colonic enema containing Cat-S or vehicle.

RESULTS: Cat-S and trypsin induced hyperexcitability of colonic DRG neurons. Action potential numbers increased by > 150%; p = 0.003 and 81%; p = 0.02 in Cat-S and trypsin treated neurons, respectively. In addition, trypsin decreased the rheobase by > 55%; p = 0.0001. E-64 inhibited only Cat-S induced hyperexcitability but not that of trypsin. Exposure of colonic DRG neurons to post-infected C. rodentium supernatants resulted in marked neuronal hyperexcitability that was reversed by E-64. The
rheobase decreased by ~ 35% (p = 0.009) and action potential discharges increased by > 123% (p = 0.05) in post-infected C. rodentium supernatant treated neurons. In addition, intra-colonic administration of Cat-S induced visceral hypersensitivity in response to CRD. When colonic DRG neurons were incubated with human IBS supernatants in the presence of E-64 and compared to human IBS or control supernatants alone, neuronal excitability induced by IBS supernatants was blocked by the inhibitor. CONCLUSION: These studies demonstrate that Cat-S selectively evokes hyperexcitability of colonic DRG neurons and enhances visceral hyperalgesia. In addition, the inhibition of Cat-S activity in post-infected C. rodentium and IBS supernatants demonstrate that cysteine proteases are expressed in colonic tissues, and appear to be important mediators underlying the supernatants induced neuronal hyperexcitability. These findings suggest an important role for cysteine proteases in visceral nociception and hypersensitivity in human IBS.
INTRODUCTION

A characteristic feature of PI-IBS, a subtype of IBS that can develop following an acute episode of infectious gastroenteritis (Marshall et al., 2006; Spiller & Garsed, 2009a), is hypersensitivity of visceral afferent fibers (Barreau et al., 2008; Hughes et al., 2009a). This hypersensitivity takes two forms; hyperalgesia, which is the reporting of exaggerated pain in response to a given noxious stimuli, and allodynia - the reporting of pain in response to an otherwise non-painful stimuli. However, appropriate drug targets for the effective control of pain in these patients have yet to be identified because knowledge of the underlying mechanisms is limited. This lack of understanding is partly responsible for the significantly high socio-economic burden of the condition (Camilleri & Williams, 2000; Vanner et al., 1999).

Primary afferent nerve fibers, whose cell bodies are located in the dorsal root ganglion (DRG), richly innervate the colon and sense changes in the chemical milieu of their environment (Hughes et al., 2009b). Such changes can result from visceral inflammation whereby activated immune cells release a number of mediators within the vicinity of the nerve terminals, including proteases, which are capable of inducing their excitation. Proteases, including the serine and cysteine families, are known to play an important role in inflammation (Conus & Simon, 2010). To date studies investigating their role in visceral nociception have focused mainly on serine proteases (Barbara et al.,
There is practically little known about a role for cysteine proteases. Cysteine proteases are a group of enzymes with papain-like activity, found in the lysosome of mammalian cells. Currently, there are 11 members of the human cathepsin family identified and include cathepsins B, C, X, K, L F, S, H, G, O and V (Conus & Simon, 2010). This study focused on cathepsin S (Cat-S) because in contrast to other members who are ubiquitously expressed, Cat-S is specifically expressed in bone marrow-derived immune cells such as macrophages, dendritic cells and B-lymphocytes (Petanceska et al., 1996). It is also expressed in non-immune cells, such as microglia and epithelial cells (Clark et al., 2007); and whereas most secreted cathepsins are biologically active in alkaline environments, secreted Cat-S is bioactive in an acidic environment, thus enabling its actions in acidic sites of inflamed tissues to lead to tissue destruction and pain.

The mechanisms that underlie the induction of pain by Cat-S are not clear. But, a recent study suggested that Cat-S may exert excitatory effects on neurons because it was found that a Cat-S inhibitor decreased neuropathic pain in rat following peripheral nerve injury (Barclay et al., 2007;Clark et al., 2007); and when administered by intra-plantar injection in rat, provoked mechanical hyperalgesia (Barclay et al., 2007;Clark et al., 2007). However, these effects were observed in somatic pain studies; how it may impact on visceral pain signaling is not known. Therefore, we investigated here whether Cat-S
contributes to visceral hypersensitivity in patients with PI-IBS by directly activating colonic sensory nerve fibers whose cell bodies lie in the dorsal root ganglion.

METHODS

Animals

Male C57BL/6 mice of 6-8 weeks old, weighing between 25 - 30 g (Charles River Laboratories, Montreal, Quebec Canada) were used for this study. Mice were separately housed in cages in a temperature and humidity controlled environment with 12 h light and dark cycle; and had free access to standard food and drinking water ad libitum. The Queen’s University Animal Care Committee and the Canadian Council of Animal Care guidelines were followed in performing all experiments.

Fast-Blue surgeries

Surgeries for the injection of Fast-Blue (FB) dye that enabled identification of colon sensory neurons were performed as previously described (Beyak et al., 2004; Kayssi et al., 2007). Briefly, mice were anesthetized with ketamine-Xylazine (0.015 mg - 0.001 mg g⁻¹ weight) and the colon accessed through a mid-line laparotomy. The retrograde marker Fast Blue (1.7% wt vol⁻¹ in sterile water) was injected into multiple locations on the colon wall in small volumes of ~ 1 - 2 µL. Labeling of unwanted tissues was avoided by wiping off excess dye from
injection sites. The colon was placed back into the abdominal cavity and the incision wound closed. Mice were left to recover on a warm bed, monitored for signs of distress and, were allowed to feed and drink ad libitum.

**Isolation of ganglia from thoracic vertebrae T₉ to T₁₃**

To isolate the DRGs, mice were sacrificed seven days after FB surgeries by exsanguinations following intra-peritoneal injection of combined Ketamine-Xylazine (0.02 mg - 0.002 mg ml⁻¹) anesthesia. The thoracic chamber was exposed and intra-cardiac perfusion performed through the left ventricle with 30 ml ice-cold Ca²⁺/Mg²⁺ free HBSS for ~ 3 minutes. Heparin (0.2 ml) injected into left ventricle was used to prevent clot formation and a small incision made in the right atrium allowed proper drainage of blood. Then the vertebral column was dissected out and thoracic vertebra T₉ to T₁₃ ganglia isolated bilaterally and placed in ice-cold HBSS.

**Preparation of primary DRG cultures**

DRG neurons from isolated ganglia were prepared using a two step enzymatic dissociation protocol as previously described (Ibeakanma & Vanner, 2010; Stewart *et al*., 2003). Briefly, isolated ganglia were first incubated in papain for 10 min at 37 °C followed by another 15 min incubation at 37 °C in collagenase (Worthington; Lakewood, NJ, USA) (1 mg ml⁻¹) and dispase (Roche; Indianapolis, IN, USA) (4 mg ml⁻¹). The partially digested ganglia were then
triturated (approximately 10 times) with a fire-polished Pasteur pipette to disperse the neurons. Dispersed neurons were washed with 2 ml warm F12 medium containing 10% FCS to stop enzyme actions. Following centrifugation at 800 x g for 1 min and careful discarding of the supernatant, neurons were re-suspended in F12 medium (pH ~ 7.4) containing 10% FCS, penicillin (100 U ml⁻¹), and streptomycin (100 µg ml⁻¹) and plated on cover slips coated with sterile Laminin-poly-D lysine (0.017 mg ml⁻¹ and 2 mg ml⁻¹) in 96-well culture plates. The plates were placed in a humidified chamber maintained at 95% O₂ and 5% CO₂ for 24 hr before being used. Following overnight cultures neurons were treated with or without 500 nM cathepsin-s (a cysteine protease) for ~ 30 – 60 min prior to being used for electrophysiological studies. For C. rodentium colonic supernatant studies, neurons were pre-incubated in the presence or absence of E-64 (100 µM; a cysteine protease inhibitor) 30 min before overnight cultures in media (500 µl) containing supernatant (250 µl).

**Preparation of colonic supernatants**

*Post-infected mouse colonic supernatants:* Under light anesthesia induced with Isoflurane (0.5 ml), 0.125 ml of C. rodentium, strain DBS 100 (1.02 x 10^{10} CFU) or distilled water was administered to mice as previously described (Ibeakanma et al., 2009). At day 30 following gavage, mice were euthanized and intra-cardiac perfusion performed. The abdominal cavity was opened, the whole colon from distal to proximal section excised, flushed with normal saline to
remove fecal pellets and weighed. The colon was cut into ~ 2 mm segments and each incubated overnight with 250 µl RPMI media in an incubator at 37 °C, 95% O₂ and 5% CO₂. After 24 h incubation, the supernatants were harvested and stored at -80 °C until when used.

**Human biopsy supernatants:** Informed consent was obtained from all patients and the Queen’s University Human Ethics Committee gave approval for protocols used. Eight pinch colonic biopsy samples were obtained from IBS- or control- patients undergoing colonoscopy at the Hotel Dieu Hospital and Kingston General Hospital, Kingston, Ontario, Canada. Samples were weighed and transferred to tissue culture well plates containing 250 µl RPMI with 10% fetal calf serum (FCS), penicillin, streptomycin, gentamicin and amphotericin-B; and incubated overnight. The supernatants were subsequently collected and stored at -80 °C until when needed.

**Electrophysiological studies**

Perforated patch-clamp studies in current clamp mode were performed on cultured nociceptive DRG neurons (sizes ≤ 40 pF) to assess their membrane electrical activities. Neurons innervating the colon were identified by their blue fluorescence emission when observed in an inverted Olympus IX70 microscope (Olympus Inc., Markham, ON, Canada) fitted with a U-MWIG2 filter, under brief exposure to ultraviolet light due to the FB dye taken up by their cell bodies. Overnight primary cultures were utilized to minimize space-clamp and size
measurement problems that could occur due to overgrowth of neural processes. Parameters of neuronal excitability, rheobase and action potential discharges at twice the rheobase, were assessed by subjecting neurons to a 500 ms duration pulse injection protocol in 10 pA increments. The resistance of pipettes used was between 2-5 MΩ when introduced into the bath solution. Pipettes were pulled from thin walled glass capillary tubes (Warner Instruments), with no internal filament, using a Narishige PP-830 puller, and polished with a Narishige MF-830 micro forge (Narishige Group, Japan). Membrane currents were recorded from neurons using Axopatch 200B amplifiers and acquired with Digidata 1322A (Axon instruments, Union City, CA, USA.). Currents were low-pass filtered at 5 kHz, acquired at 20 kHz and processed using Clampfit 10.2 software (Axon Instruments, Union City, CA, USA.). Data were analyzed using Prism 5 software (GraphPad, San Diego, CA, USA).

**Solutions**

The compositions of the standard solutions used are (in mM): Bath solution – 140 NaCl, 5 KCl, 10 HEPES, 10 Glucose, 1 MgCl₂, and 2 CaCl₂; pH adjusted to 7.4 with NaOH. Pipette solution – 110 K-Gluconate, 30 KCl, 10 HEPES, 1 MgCl₂, and 2 CaCl₂; pH adjusted to 7.25 with KOH. A continuous flow of external solution at a rate of 2 ml min⁻¹ was maintained in the recording chamber during recording. Electrical connection between recording electrode and intracellular molecules was achieved through perforations made on the cell.
membranes by Amphotericin-B (240 µg ml⁻¹) (Sigma Aldrich, St Louis, MO, USA) dissolved in pipette solution. The Amphotericin-B mixed internal solutions were replaced every 2 – 3 h to maintain the activity of the compound which breaks down rapidly on light exposure. All studies were conducted at room temperature (~23°C) and the following parameters of neuronal excitability measured - changes in the resting membrane potential, rheobase, action potential numbers at twice the rheobase and input resistance.

**Colorectal distension (CRD)**

Mice were anaesthetized by IP injection of 0.1 ml 10 g⁻¹ body weight of combined xylazine-ketamine (70% distilled H₂O, 25% Ketamine plus 5% Xylazine) and underwent surgical implantation of 3 groups of electrodes (Bioflex insulated wire AS631; Cooner Wire, Chatsworth, CA, USA) into the external oblique muscle of the abdominal wall. Electrodes were passed under the skin and exited at the back of the neck through protective plastic tubings attached to the skin. After surgeries, animals were monitored daily for signs of discomfort and dehydration.

Four days following surgical implantation of electrodes, CRD was performed as previously described (Cenac et al., 2007; Larsson et al., 2003). Briefly, mice were placed in restraining devices (modified falcon tube) that prevented them from escaping, moving or turning around for a control period of 30 min. The devices were in turn secured to the work-bench using adhesive
tapes. To reduce stress reactions during experiments, mice were habituated to the procedure 1 h each day for a total of 3 days prior to the day of CRD experiment. CRD was performed using wet (in-house made) balloons (10.5 mm diameter) inserted into the colon (5 mm proximal to the rectum); with 15 mmHg incremental inflations from 0 to 60 mmHg. Distensions, lasting 10 s, were performed 3 times for each pressure of 15, 30, 45, and 60 mmHg with 4 min between each distention.

Mice were given 100 µl enema of 1 µM Cat-S solution (50 µl Cat-S plus 50 µl 35% EtOH) under light isoflurane anesthesia. Control animals received intra-colonic administration of 100 µl of a saline solution (50 µl normal saline plus 50 µl 35% EtOH). The enema was administered twice to each mouse at 4 h intervals and CRD commenced 30 min following the last administration.

**Materials and drugs**

Stock solutions of Cat-S were generously provided by Dr. Nigel Bunnett and were stored at -80 °C until needed. Further dilutions were made in RPMI culture media to a final concentration of 500 nM. E-64 (Calbiochem/EMD Biosciences, Inc. La Jolla, CA, USA) was reconstituted to a stock concentration of 5 mg mL⁻¹ DMSO; aliquots were made and stored at -20 °C. When needed, aliquots were thawed and further dilutions made to working concentrations of 100 µM. HBSS, RPMI and F12 medium were purchased from Gibco (Invitrogen Corporation, Van Allen Way Carlsbad, CA, USA). Poly-D lysine was bought from
VWR (West Chester, Pennsylvania, USA), while papain was from Worthington (Lakewood, NJ, USA). All other substances were purchased from Sigma-Aldrich (Oakville, ON, Canada).

**Statistical analysis and data expression**

Data analysis was performed using PRISM 5 (GraphPad, San Diego, CA, USA) and are presented as mean ± SEM, with n referring to number of neurons analyzed. Significant difference between groups was determined by one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison *post-hoc* tests and, between two conditions with unpaired student’s t-test. A p-value of <0.05, 0.01 or 0.001 was accepted as significant and is indicated as *, ** and *** respectively.

**RESULTS**

**Recombinant human Cat-S induces hyper-excitability of colonic mouse DRG neurons that is reversible when neurons are pretreated with E-64**

To examine whether Cat-S participates in pain associated with post-infectious conditions of the bowel (e.g. PI-IBS), we first determined whether it can directly activate colon sensory DRG neurons. Perforated patch recordings in current-clamp mode were obtained from controls, Cat-S- and Cat-S plus E-64-incubated (30 min) colonic DRG neurons. As illustrated in figure 4-1A and B, neurons incubated in Cat-S (500 nM) induced increased neuronal excitability that
was attenuated when the neurons were pre-exposed for 30 min to cysteine protease specific inhibitor E-64 (100 µM). The action potential number increased by more than 153% and 100% in neurons exposed to Cat-S (n = 11; p = 0.003) compared to control (n = 12) and Cat-S plus E-64 (n = 14) treated cells, respectively (Figure 4-1B, right). Relative to controls, the rheobase was unaffected in Cat-S and Cat-S plus E-64 groups (Figure 4-1B, left). These data suggest that Cat-S is capable of directly activating afferent nerve terminals within their immediate surroundings to cause pain.

**Intra-colonic administration of Cat-S induced visceral hypersensitivity in response to CRD**

The majority of IBS patients report hypersensitivity symptoms in response to balloon distension of their colo-rectum (Ritchie, 1973; Whitehead *et al.*, 1990). Whether cysteine proteases participate in the generation of hypersensitivity symptoms is not known. To address this question and establish a role for cysteine proteases in visceral nociception, we compared visceral motor responses to colorectal distension in mice that received intra-colonic enema of Cat-S (100 µl of 1 µM Cat-S; 50 µl Cat-S plus 50 µl 35% ethanol) or vehicle (100 µl of mixed 50 µl normal saline and 50ul 35% ethanol). Two hours following the intracolonic administrations, VMR to CRD were markedly increased in mice that received Cat-S compared to vehicle. Significant differences in VMR were
A

Rheobase

Control

88 pA
500 ms

0.03 V
0.1 s

Cat-S

75 pA
500 ms

Cat-S plus E-64

80 pA
500 ms

2x Rheobase

172 pA
500 ms

150 pA
500 ms

160 pA
500 ms

B

Mean rheobase (pA)

Control | Cat-S | Cat-S + E-64

(12) | (11) | (14)

Mean # of action potentials at 2x rheobase

Control | Cat-S | Cat-S + E-64

(12) | (11) | (14)
Figure 4-1 Recombinant human cathepsin-S induces hyperexcitability of mouse colonic DRG neurons. (A) Representative traces of action potentials elicited by a 500 ms current injection at rheobase and twice the rheobase in control, Cat-S (500 nM) and Cat-S plus E-64 (100 µM; cysteine protease inhibitor) incubated neurons. (B) Graph summarizing effects of 30 min Cat-S treatment, in the presence or absence of E-64, on excitability of nociceptive DRG neurons. Cat-S did not alter the rheobase (left graph), but induced multiple action potentials that were attenuated when neurons were previously exposed for 30 min to E-64 (right graph). Data are presented as mean ± SEM; number in parenthesis represents the number of neurons recorded in each group, **p < 0.01, one-way ANOVA.
Figure 4-2 Visceromotor responses (VMR) to graded colorectal distension (CRD) in mice in response to intracolonic enema of Cat-S. Intracolonic administration of cathepsin-S (100 µl of 1 µM Cat-S; 50 µl Cat-S plus 50 µl 35% ethanol) increases visceral sensitivity to CRD at 15, 45 and 60 mmHg (white squares) compared to controls (black circles) that received vehicle (100 µl of mixed 50 µl normal saline and 50 µl 35% ethanol) only. Data expressed as mean ± SEM, n = 5 mice per group, *P < 0.05 (two-way ANOVA with bonferoni post test correction).
observed at noxious distension pressures of 15, 30, 45 and 60 mmHg, suggesting exaggerated hyperalgesic response and allodynia (Figure 4-2).

**Effects of trypsin on colonic DRG neurons are not altered by E-64**

To determine whether E-64 inhibitor is specific to cysteine proteases, neuronal excitability was assessed in a set of experiments in which neurons were exposed for 30 min to trypsin (n = 19), a serine protease, trypsin plus E-64 (n = 17) or control media only (n = 22). As shown in figure 3, trypsin decreased the rheobase by more than 55% (p = 0.0001) (Figure 4-3, upper left), depolarized the membrane by ~ 5% (p = 0.008) (Figure 4-3, lower left) and increased the number of action potential discharges at twice the rheobase by ~ 82% (p = 0.02) (Figure 4-3, upper right). However, these effects were unaffected by the presence of E-64, confirming that effects of the inhibitor are specific to Cat-S but not trypsin. There was no change in the input resistance (Figure 4-3, lower right).

**Cysteine proteases play a prominent role in the excitatory actions of post-infected C. rodentium supernatants on colonic DRG neurons.**

Next we examined the effects of supernatants obtained from post-infected C. rodentium colonic tissues on the electrical properties of DRG neurons. These assessments were made in the presence or absence of E-64 in order to determine whether cysteine proteases are important factors mediating their actions. Interestingly, the post-infected C. rodentium supernatant caused marked
Figure 4-3 Effects of cysteine protease inhibitor, E-64, on trypsin-induced neuronal hyperexcitability. Graph summarizing effects of trypsin (a serine protease) on the electrical properties of colonic nociceptive DRG neurons, in the presence or absence of E-64 (100 µM). Trypsin induced marked changes in rheobase (upper left graph), action potential discharge at twice the rheobase (upper right graph) and resting membrane potential (lower left graph) that were not affected by the presence of E-64. Data reported as mean ± SEM; number in parenthesis indicates number of neurons recorded from; *p < 0.01, ***p < 0.001, one-way ANOVA.
increases in excitability of colonic DRG neurons that was reversed by a 30 min pre-incubation with E-64 compared to the matching controls (Figure 4-4A). The rheobase in *C. rodentium*-supernatant incubated neurons (n = 15) decreased by 65% compared to control saline-supernatant (n = 15; p = 0.009); an effect blocked in *C. rodentium*-supernatant plus E-64 pre-incubated neurons (n = 13) (Figure 4-4A, left). Also, the action potential discharge at twice the rheobase increased in *C. rodentium*-supernatant by more than 123% (n = 15; p = 0.048) compared to controls (n = 15), an effect also reversed in the presence of E-64 (n = 13) to comparable control values (Figure 4-4A, right). There was no difference between the control saline-supernatant and saline-supernatant plus E-64 groups suggesting that E-64 by itself does not alter the intrinsic properties of the neurons. In a similar fashion, rheobase did not differ between the groups (Figure 4-4A, left).

Cysteine proteases are important mediators underlying IBS-supernatant induced hyperexcitability of colonic nociceptive DRG neurons

To establish the relevance of the above findings in humans, we tested whether IBS-supernatant alters the excitability of colonic DRG neurons, and then examined whether cysteine proteases make any contributions to such actions. To do this, we conducted patch clamp studies on neurons incubated with IBS supernatants in the presence of E-64 and compared results with IBS- or control-supernatant incubated neurons (Figure 4-4B). Action potential discharges in IBS-
supernatant incubated neurons increased by more than 100% (n = 8; p = 0.049) compared to control neurons incubated in control patient supernatant (n = 6) (Figure 4-4B, right). E-64 reversed this effect to comparable control level (n = 9). The rheobase did not differ between the three groups (Figure 4-4B, left). Taken together, these data demonstrate that supernatant from colonic biopsies of IBS patients increase excitability of colonic DRG neurons, and that cysteine proteases are important mediators of the supernatant effects.

DISCUSSION

This study tested the hypothesis that cysteine proteases contribute to visceral pain signalling in PI-IBS through direct activation of colonic nociceptive DRG neurons. Consistent with our hypothesis, Cat-S, an important member of the secreted lysosomal cysteine proteases, induced hyperexcitability of colon specific DRG neurons and, induced visceral hyperalgesia and allodynia when introduced intra-colonically in mice. In addition, we show here for the first time using human colonic IBS- and mouse post-infectious C. rodentium- supernatants that cysteine proteases are important factors mediating excitatory actions of the supernatants on colonic DRG neurons. This suggests an important role for lysosomal cysteine proteases in inflammation-induced alterations of peripheral pain signaling. Taken together, these findings are relevant because treatment strategies targeted at disrupting abnormal sensory signaling by cysteine proteases could prove helpful in pain management in PI-IBS.
Supernatants from human IBS and post-infected C. rodentium colonic tissues each mimic effects of recombinant human Cat-S on colon specific mouse DRG neurons. (A) Graph demonstrating the effects of colonic supernatants from post-infected C. rodentium (citro-sup) and control (saline-sup) tissues, in the presence (+) or absence (-) of E-64 (100 µM), on the electrophysiological properties (rheobase and action potential number at twice the rheobase) of nociceptive colonic DRG neurons. (B) Graph illustrating induction of hyperexcitability of nociceptive DRG neurons by human IBS-patients supernatant and its reversal in neurons previously exposed to E-64. Data reported as mean ± SEM. *p < 0.05, one-way ANOVA.
There has been increasing interest in the role of proteases in visceral pain expression, following the findings of increased levels of the enzymes in colonic tissues of both human and animal models of IBS, including PI-IBS (Barbara et al., 2004b; Cenac et al., 2007; Clarke et al., 2009; Spiller & Garsed, 2009a). Proteases, including the serine and cysteine families, are secreted by activated mast cells, immune cells and enterocytes, and are known to play an important role in inflammation (Conus & Simon, 2010). To date studies investigating their role in visceral nociception have focused mainly on serine proteases (Barbara et al., 2007; Cenac et al., 2007). There is very little known about a role for cysteine proteases. A recent study however suggested that Cat-S, a member of the cysteine protease family, may exert excitatory effects on neurons because it was found that a Cat-S inhibitor decreased neuropathic pain in rat following peripheral nerve injury (Barclay et al., 2007; Clark et al., 2007). Also that intra-plantar injection of rat recombinant Cat-S evoked mechanical hyperalgesia (Barclay et al., 2007; Clark et al., 2007). But these effects were observed in somatic pain studies; how it may impact on visceral pain signaling is not known. Based on these studies, we conducted electrophysiological studies on colon specific DRG neurons identified by retrograde labeling following Fast-Blue dye injection into the colon wall. We found significantly increased action potential discharges at twice the rheobase in neurons exposed to Cat-S. Interestingly, these effects were reversed by prior exposure of the neurons to E-64. Unlike with Cat-S, E-64 had no effects on trypsin (a serine protease) induced neuronal hyperexcitability,
demonstrating that effects of the inhibitor are specific to cysteine- but not serine-proteases. This novel finding is the first demonstration of a direct induction of neuronal hyperexcitability of colonic nociceptive DRG neurons by any member of the cysteine proteases.

The consistent finding of low levels of inflammation characterized by presence of increased numbers of activated immune and mast cells in colonic mucosa of both human and animal models of PI-IBS (Barbara et al., 2004b; Lee et al., 2008) suggest cysteine protease activity could be elevated in these tissues, thus enabling perpetuation of persistent pain signaling. We tested this notion by examining whether the specific cysteine protease inhibitor, E-64 blocks the actions of supernatants from post-infected animals and by so doing, determined whether they participate in the alteration of neural excitability. We found that colonic post-infected C. rodentium supernatant evokes neuronal hyperexcitability in a fashion similar to exogenous Cat-S. The fact that the effects were completely reversed by E-64 suggests a prominent role for cysteine proteases in the actions of the supernatants. E-64 by itself does not affect the intrinsic properties of the neurons since no differences were observed between the E-64 treated and non-treated control groups. These findings support a role for cysteine proteases contained in the representative supernatants in alteration of neural excitability. Moreover, VMR responses to colon distension was significantly greater in mice that received intracolonic administration of Cat-S,
suggesting exaggerated hyperalgesic response to a given colonic distension stimuli.

Further support of a role for cysteine proteases in visceral pain signaling was provided by our human IBS data. These data show for the first time that blockage of cysteine protease activity with a specific cysteine protease inhibitor reverses the excitatory actions of IBS supernatant on nociceptive colonic sensory neurons. Previous studies that examined the importance of proteases in visceral pain signaling in IBS focused on serine but not cysteine proteases (Barbara et al., 2007; Cenac et al., 2007). These studies established that pro-nociceptive actions of IBS supernatants were also compromised in presence of global protease inhibitors, thus suggesting that other classes of proteases could be important signaling molecules in the alteration of pain signaling in IBS.

The mechanisms that underlie nociceptive actions of Cat-S are not clear, but involvement of PARs signaling was suggested by a recent study that found that Cat-S activated calcium influx in HEK cell transfected with PAR2 & PAR4 (Reddy et al., 2010). Previous studies have established pro-nociceptive and anti-nociceptive roles for PAR2 and PAR4, respectively (Karanjia et al., 2009; Kayssi et al., 2007); and since PAR2 is expressed on colonic tissues and colon projecting DRG neurons, it’s possible that the cysteine protease-induced neuronal hyperexcitability, as well as visceral hypersensitivity could be mediated through direct interaction with PAR2. Further studies are needed to clarify this matter.
In conclusion, our study has provided direct evidence in both human and animal models of IBS that support a major role for cysteine proteases in visceral nociception. These findings may have an important impact on clinical settings of PI-IBS since signaling by cysteine proteases could potentially be novel therapeutic target in abdominal pain management in patients with PI-IBS. Additional studies are needed to determine whether these enzymes have similar effects on pain signaling in IBD and whether the mechanisms involve direct activation of PARs. Furthermore, understanding the identity and mechanisms of actions of other proteases that possibly contributed to these effects could help increase our knowledge of the pathogenesis and identify biomarkers for subtypes of IBS.
CHAPTER 5: TNF-α IS A KEY MEDIATOR OF THE PRO-NOCICEPTIVE EFFECTS OF MUCOSAL SUPERNATANT FROM HUMAN ULCERATIVE COLITIS ON COLONIC DRG NEURONS
ABSTRACT

Objectives: Abdominal pain is a serious cause of morbidity in patients with inflammatory bowel disease. To better understand the mechanisms and potentially identify new targets for treatment we examined the effects of inflammatory supernatant from colonic biopsies of patients with active ulcerative colitis on mouse colonic nociceptive dorsal root ganglia neurons. Methods: Acutely dissociated dorsal root ganglia neurons innervating the mouse colon were incubated in supernatants obtained from colonic biopsies from UC patients. Whole-cell patch clamp recordings were obtained to examine the effects on neuronal excitability. The role of TNF-α was studied using TNF-α receptor (TNFR) knockout mice and comparing supernatant and TNF-α actions. Results: UC-supernatants significantly decreased the rheobase and increased action potential discharge, indicating increased neuronal excitability. Human biopsies exhibited high levels of TNF-α and mouse colonic neurons only exhibited TNF-α receptor 1 mRNA. Incubation with TNF-α recapitulated the supernatant effects on neuronal excitability and supernatant and TNF-α actions were almost completely blocked in TNFR knockout mice. In voltage clamp studies transient I_A and I_K currents were suppressed and Nav 1.8 currents were enhanced by TNF-α and UC-supernatant, suggesting that multiple underlying mechanisms contributed to the enhanced excitability. Conclusions: UC-supernatants enhance neuronal excitability of sensory DRG neurons innervating the colon. TNF-α is a key mediator which acts at neuronal TNF-α receptor 1 to modulate Kv and Nav...
currents. Together these data provide a number of potential new targets for pain management in UC.
INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn’s disease and ulcerative colitis (UC), are common disorders that affect over 1 million North Americans (Beyak & Vanner, 2005; Papadakis & Targan, 1999; Papadakis & Targan, 2000). This chronic inflammatory condition typically has a relapsing and remitting course characterized by bloody diarrhea, fatigue and abdominal pain. These symptoms are a major cause of morbidity and can severely limit quality of life. Current treatment of abdominal pain is largely limited to narcotic analgesics such as morphine, but these have important side effects, including altered cognition, drowsiness, decreased energy levels, and nausea and vomiting. Furthermore, in patients with active colitis, they can precipitate life threatening toxic megacolon (Gan & Beck, 2003). As a result, there is a great need to better understand the mechanisms underlying pain in patients with IBD and to identify new therapeutic targets.

Chemical and mechanical stimuli in the colon are detected by axon terminals of dorsal root ganglia (DRG) neurons which relay signals to the CNS (Holzer et al., 2001; Junger & Sorkin, 2000). During colitis, such as a UC flare-up, a large number of pro-inflammatory mediators are released within the intestinal wall and the serum, which can elicit pain by activating specialized receptors on the Aδ and C fiber nociceptors (Bueno & Fioramonti, 2002; Shanahan & Targan, 1992). Assays of inflamed mucosa in UC and CD have increased protein and mRNA of a number of cytokines, including IL-1β, IL-6, IL-8 and TNF-α (Fiocchi,
1998; Present et al., 1999; Sartor, 1994). There is evidence many of these mediators can sensitize channels, such as the transient receptor potential (TRPs) resulting in enhanced neurotransmission produced by mechanical, chemical and other stimuli (Cunha et al., 1992; Khan et al., 2008; Obreja et al., 2005; Woolf et al., 1997). Furthermore, they can also modulate Na_v and K_v ion channels which underlie the generation of the action potentials (Czeschik et al., 2008; Matsutomi et al., 2006; Rush & Waxman, 2004; Viviani et al., 2007) thus also increasing pain signaling from a broad range of sensory stimuli. Recent evidence suggests that anti-nociceptive mediators are also released (Auge et al., 2009; Verma-Gandhu et al., 2007), which may be dependent on the inflammation’s type (e.g. IBD vs. infectious) and progression (e.g. acute vs. chronic). Therefore the net effect of the inflammatory milieu on neuronal excitability may depend upon the balance of these factors.

Amongst the pronociceptive mediators, TNF-α is thought to play an important role. When applied subcutaneously or perineurally, TNF-α lowers mechanical activation threshold in nociceptors, rapidly evokes ongoing activity in C-fibers, and elicits mechanical allodynia and/or hyperalgesia (Junger & Sorkin, 2000; Reeve et al., 2000). However, it is unknown whether colonic DRG neurons are affected similarly, and whether any effects on neuronal excitability make a significant contribution within the wider inflammatory cytokine milieu of IBD.

The ability to obtain a representative supernatant from mucosal biopsies from patients with active UC allows the study of the net effect of this inflammatory
milieu on nociceptive neurons. We found that these supernatants markedly increased the excitability of mouse colonic nociceptive neurons. Using combined *in vitro* electrophysiological and molecular techniques we sought to determine the role that TNF-α may play in these actions and to examine voltage-gated ion channels which underlie these changes in excitability.

**MATERIALS AND METHODS**

**Human biopsy samples**

A total of 136 colonic tissue samples were obtained from 17 patients (8 UC and 9 controls) undergoing colonoscopy at the Hotel Dieu Hospital and Kingston General Hospital, Kingston, Ontario, Canada. The protocol was approved by the Queen’s University Human Ethics Committee, all patients gave informed consent. UC diagnosis was based upon endoscopic and histological criteria. Control biopsies were from patients undergoing colon cancer screening; none exhibited endoscopic signs of inflammation. In patients with active UC, eight adjacent biopsies were collected from the region of inflamed colon. The samples were weighed and transferred into well plates of 250 μl RPMI with 10% FCS, Penicillin/Streptomycin and Gentamicin/Amphotericin B and incubated overnight at 37 °C, under 95% O₂ / 5% CO₂. Subsequently the supernatants were collected and stored at -80 °C for further use.
Retrograde labeling and isolation of colon-projecting neurons

All animal protocols were approved by the Queen’s University Animal Care Committee. Adult Mice (CD1) 5–6 weeks from Charles River Laboratories (Montreal, QC, Canada) of either sex were used for all studies. Colon-projecting DRG neurons were labeled using Fast Blue (Cedarlane Laboratories; Homby, ON) 7 days in advance as previously described (Beyak et al., 2004). For DRG isolation, mice were anesthetized with isoflurane and killed by cervical transection. The spinal cord was removed and DRG from T9 to T13 were dissected, as these levels receive nociceptive input from the colon (Traub, 2000). The DRG neurons were acutely dissociated as previously described (Moore et al., 2002; Stewart et al., 2003). Dispersed neurons were suspended in 500 μl of RPMI medium containing 10% fetal bovine serum, 100 U/ml Penicillin, 0.1 mg/ml Streptomycin, and 2 mM glutamine and plated on Pure-Col (Inamed Biomaterials, Fremont, CA, USA) coated cover slips (60 μl/ml). To this was added 250 μl of either UC patient or control supernatant, or a human TNF-α solution (final concentration: 100 ng/ml) (Sigma Aldrich, St. Louis, MO) or RPMI media alone, for 24 hours in a humidified incubator at 37 °C, 95% air and 5% CO2 before electrophysiological studies.

Electrophysiological recordings

Current or voltage patch clamp experiments were performed on Fast Blue labeled neurons. Small neurons (≤ 40 pF capacitance) were studied since they
are thought to be nociceptors (Beyak & Vanner, 2005; Gold et al., 1996; Moore et al., 2002; Yoshimura & de Groat, 1999). Signals were amplified by an Axopatch 200B amplifier filtered at 5 kHz and digitized at 20 kHz with a Digidata 1322A A/D converter and analyzed using Clampex 10.0 (all from Axon Instruments, San Jose, CA).

**Solutions**

For perforated-patch current-clamp recordings with Amphotericin B (Sigma Aldrich, St. Louis, MO), the following solutions were used (mM): (External), 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, and 10 D-glucose, pH adjusted to 7.4 with NaOH. (Pipette) 110 K-Gluconate, 30 KCl, 10 HEPES, 1 MgCl2, and 2 CaCl2 with pH adjusted to 7.25 using KOH. Perforated-patch voltage-clamp recordings of potassium currents were obtained in sodium-free bath solution; (mM) 140 NMDG, 4 KCl, 1.8 HEPES, 1 Dglucose, 1 CaCl2, 1 MgCl2, pH 7.4 adjusted with HCl. For whole-cell voltage clamp recording of sodium currents, the patch pipette solution contained (mM): 10 NaCl, 110 CsCl, 3 MgCl2, 10 HEPES, 0.6 Na-GTP, 3 ATP and 10 EGTA, pH adjusted to 7.3 with CsOH. Bath solution (mM): 55 NaCl, 90 TEA–Cl, 1 CaCl2, 2 MgCl2, 0.1 CdCl2, 10 HEPES, 5 Glucose, and 5 4-aminopyridine (4-AP), pH adjusted to 7.4 with NaOH. The liquid junction potential was compensated for by adjusting the zero current potential.
Potassium current recordings

$I_A$ and $I_K$ currents were separated as previously described (Ibeakanma et al., 2009; Stewart et al., 2003). Briefly, total potassium current was elicited using 500 ms voltage steps from -90 mV to +50 mV in 10 mV increments from a holding potential of -100 mV. $I_K$ was then elicited using the same pulse protocol, but from a holding potential of -60 mV to inactivate $I_A$. Amplitude of the $I_K$ current was measured at 450 ms at each voltage step (see Figure 5-5). $I_A$ was isolated by subtracting $I_K$ from the total potassium current. The amplitude of the $I_A$ current was measured at the peak of the transient component at each voltage step (see Figure 5-5). These voltage protocols may result in a small cross-over contamination of the two currents, but previous studies in our lab suggest that this contamination would not significantly alter the interpretation of the results (Stewart T et al, 2003). Activation and inactivation curves were analyzed as previously described (Ibeakanma et al., 2009; Stewart et al., 2003).

Na$_v$ current recordings

Membrane currents were recorded using the whole-cell patch-clamp technique and activation and inactivation curves analyzed as previously described (Ibeakanma et al., 2009; Stewart et al., 2003). Cells were held at a holding potential of -120, or -100 mV and then stepped from -80 to +35 mV in 10 mV intervals for 200 ms each. 60-70% series resistance compensation was applied. Only cells with adequate voltage- and space-clamp (Ogata &
Tatebayashi, 1993) and with gradual Na+ current activation were used. 1 μM TTX was used to isolate TTX-R Nav1.8 currents. TTX-R Nav1.9 currents should have an activation threshold about 20 mV more negative than the threshold for TTX-R Nav1.8 currents (Maruyama et al., 2004), however, none were seen.

**ELISA measurement of TNF-α content of supernatants**

Human TNF-α enzyme-linked immunosorbent assays (ELISA) kits (Medicorp Inc., Montréal, QC, Canada) were used to assay supernatants and standards simultaneously as per manufacturer’s instructions. Polyclonal goat anti-human TNF-α antibodies were used as capturing antibodies and biotinylated polyclonal rabbit anti-human TNF-α antibodies as detecting antibody. Streptavidin-HRP and stabilized chromogen were added as colour indicators. Optical densities of plates were read at 450 nm in a Titertek Multiskan Plus spectrophotometer right after color reactions were stopped with acid. All steps were performed at room temperature and samples were assayed in duplicates. Results are expressed as pg/mg tissue wet weight.

**TNF-α receptor mRNA amplification**

Total RNA was isolated from laser-captured Fast Blue labeled colonic DRG neurons using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription of 0.8 μg of RNA was performed using Expand RT (Roche - Mannheim, Germany) and Oligo dT12-18
(Invitrogen – Carlsbad, CA). 2 μl of each reverse transcript served as a template for polymerase chain reaction (PCR) amplification to estimate the mRNA expression of TNFR1 and TNFR2 using Platinum Taq DNA Polymerase (Invitrogen – Carlsbad, CA). Primers used for analysis were as follows: TNFR1 Sense 5’- GGA TTG TCA CGG TGC CGT TGA AG-3’, and Antisense 5’- TGA CAA GGA CAC GGT GTG TGG C-3; TNFR2 Sense 5’- AAC GGG CCA GAC CTC GGG T-3’, and Antisense 5’- AGA GCT CCA GGC ACA AGG GC-3’.

Amplification conditions were 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C. After 35 cycles of reaction, which preliminary studies showed was within the linear range for these primers and samples, 5 ml of each amplified fragment and 1 μl loading buffer were subjected to 1.5% agarose gel (Bioshop - Burlington, ON) electrophoresis separation and stained with ethidium bromide (Fisher – Ottawa, ON). Experiments were repeated five times.

Statistics

All data are expressed as mean ± SEM. Treatment effects were analyzed by Student’s test using Prism (GraphPad, San Diego, CA, USA). One- or two-way ANOVA with Bonferoni correction was applied for comparison of multiple groups. Fitting of activation / inactivation data was done using the Boltzmann equation fit function in Origin 6.0 (Microcal). Mean voltages of half activation (Vₜ) and slope factors (k) were obtained from individual Boltzmann curve fits.
RESULTS

Patient characteristics

Mucosal biopsies were obtained from regions of colonic inflammation in 8 patients with active UC (see Table 5-1). Control biopsies were obtained from 9 patients undergoing colonoscopy for colon cancer screening. None of the patients had complaints of diarrhea and the mucosa had a normal endoscopic appearance.

Ulcerative colitis supernatant induces hyperexcitability of mouse nociceptive DRG neurons

Fast Blue labeled mouse DRG neurons exhibited increased excitability following overnight incubation in UC-supernatant (representative traces shown in Figure 5-1A). The rheobase decreased by 51.7% and 55.6% in neurons incubated in the UC-supernatant (n = 15) compared to those incubated in control supernatant (n = 14, p < 0.05) and control media (n = 15, p < 0.05) respectively. The number of action potentials elicited at twice rheobase was also increased by 115.4% and 87.5% compared to control supernatant (n = 14, p < 0.001) and control media (n = 15, p < 0.001) respectively. There was no difference in the mean input resistance or the resting membrane potential between neurons incubated in UC-supernatant and control supernatant or control media. There also was no difference in excitability between neurons incubated in control supernatant and those incubated in control media alone (Figure 5-1B).
Table 5-1 Patient Characteristics

<table>
<thead>
<tr>
<th>Age</th>
<th>Duration of Illness</th>
<th>Medications</th>
<th>Biopsy proven UC</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>4 months</td>
<td>none</td>
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</tr>
<tr>
<td>25</td>
<td>3 weeks</td>
<td>none</td>
<td>yes</td>
</tr>
<tr>
<td>57</td>
<td>years</td>
<td>5-ASA</td>
<td>yes</td>
</tr>
<tr>
<td>26</td>
<td>6 months</td>
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<td>yes</td>
</tr>
<tr>
<td>59</td>
<td>years</td>
<td>5-ASA</td>
<td>yes</td>
</tr>
<tr>
<td>60</td>
<td>6 months</td>
<td>none</td>
<td>yes</td>
</tr>
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</tr>
<tr>
<td>34</td>
<td>2 months</td>
<td>steroids</td>
<td>yes</td>
</tr>
</tbody>
</table>

5-ASA, 5-aminosalicylic acid; UC, ulcerative colitis.

**TNF-α receptor expression in mouse colonic DRG neurons and TNF-α concentration in human biopsy supernatants**

To demonstrate that TNF-α receptors are expressed on the T<sub>9</sub>-T<sub>13</sub> level mouse colonic DRG neurons electrophysiologically recorded from, we profiled the expression of TNFR1 (Figure 5-2A, left gel) and TNFR2 (Figure 5-2A, right gel) in whole DRGs by RT-PCR and confirmed the presence of both transcripts. However, using retrograde Fast Blue labeling and laser capture microdissection, we found transcripts for TNF-α receptor 1 alone (Figure 5-2B). The absence of signal in the ‘no template’ samples show that there is no genomic or other DNA contamination. Supernatants obtained from ulcerative colitis patients were found by ELISA to contain > 6-fold higher TNF-α levels (41.7 ± 9.5 pg/mg tissue) compared to control patient supernatant (6.5 ± 3.4 pg/mg tissue; p < 0.007; n = 6) (Figure 5-2C). In a separate series of experiments, we measured TNF-α in
Figure 5-1 Supernatant from ulcerative colitis (UC) patient tissue induces hyperexcitability of mouse nociceptive DRG neurons. (A) Representative perforated current clamp recordings in response to a 500 ms current injection at rheobase (left) and 2x rheobase (right) from neurons incubated in UC-supernatant, control supernatant and control media. (B) Summary data illustrating the effects of incubation of DRG neurons with supernatant from ulcerative colitis patient tissues (black bar), control patient tissues (gray bar) and control media (white bar). Data represents mean ± SEM. *P < 0.05, **P < 0.001, one-way ANOVA with Bonferroni multiple comparison test.
ng/ml in the supernatant using the human TNF-α (Sigma) employed in the electrophysiological studies as the standard for the ELISA measurements and found that the supernatant TNF-α values ranged from 0.4 – 1.6 ng/ml (mean = 0.75 ± 0.18 ng/ml).

**TNF-α increases neuronal excitability of mouse colonic DRG neurons**

To examine the role of TNF-α in the action of UC-supernatant, we incubated mouse colonic DRG neurons in TNF-α (100 ng/ml). As we found with UC-supernatant incubated neurons, incubation with TNF-α (n = 15) resulted in a significant decrease in rheobase (p < 0.03) compared to control media (n = 14) (Figure 5-3). Similarly, TNF-α resulted in a > 70% increase in the number of action potentials at twice rheobase (p < 0.01) versus controls. Neither the resting membrane potential (TNF-α; -48.2 ± 1.7 mV, control; -51.04 ± 1.6 mV) nor the input resistance were significantly different between the groups. Thus, TNF-α also increases colonic DRG neuronal excitability. To ensure that the 100 ng/ml employed in the electrophysiological studies was physiological, we repeated the electrophysiological studies using a comparable TNF-α value (1 ng/ml) as that measured in the supernatants (n = 9 cells) and compared this to controls (n = 8 cells). We found that rheobase was also markedly decreased (TNF-α mean = 53.3 ± 8.0 vs. control mean = 95.0 ± 8.0 pA; p = 0.0035 and a significant increase in action potential discharge at twice the rheobase (TNF-α mean = 4.2 ± 0.6 vs. control mean = 2.0 ± 0.7; p = 0.03).
Mouse colonic DRGs express TNF-α receptor1 and TNF-α is markedly elevated in supernatants from IBD patients. (A) In whole DRG neurons, expression of both TNFR1 (120 bp product) and TNFR2 (257 bp product) was confirmed by RT-PCR and agarose gel (1.2 %) electrophoresis (arrow heads). (B) However, expression of TNFR1, but not TNFR2, mRNA was confirmed in laser captured, FB-labeled colonic DRG neurons by RT-PCR. (C) Evaluation of TNF-α level in UC (n = 6) and control (n = 6) patient supernatants using ELISA techniques. Summary bar graph demonstrating UC-supernatant contained significantly higher levels of TNF-α (black bar) compared to control patient supernatant (white bar). Values are the mean ± SEM of three independent experiments. **P < 0.005.
Figure 5-3 Exogenous tumor necrosis factor α (TNF-α) also increases excitability of nociceptive dorsal root ganglion (DRG) neurons. Data illustrating that overnight incubation with 100 ng/ml exogenous TNF-α (black bar) induced hyperexcitability of DRG neurons, causing a significant reduction in rheobase with significant increases number of action potentials at 2x rheobase and action potential half width compared to control media (white bar) incubated neurons. Membrane potential did differ between the two conditions. Data represents mean ± SEM. *P < 0.05.
The effects of UC-supernatant on neuronal excitability are markedly attenuated in TNF-α receptor knockout mice

In order to examine the contribution of TNF-α to the overall effect by UC-supernatant on DRG neuronal excitability, we studied the effects of UC-supernatant and exogenous TNF-α on neurons isolated from wild-type and TNF-α receptor knockout mice (Figure 5-4). We confirmed the presence or deletion of TNFR1 and TNFR2 in the wild-type and knockout mice respectively, by RT-PCR and agarose gel (1.5%) electrophoresis. β-Actin was used as loading control and was detected in both wild-type and knockout cDNA to confirm cDNA integrity. The absence of signal in the ‘no template’ and WT DRG RNA samples confirmed that there was no genomic DNA or other contamination (data not shown).

In control experiments using media only, we confirmed there was no difference in the intrinsic excitability of the wild type (n = 14 cells) compared to the TNFR knockout neurons (n = 13 cells). (Wild type mean rheobase = 84.3 ± 9.8 pA vs. TNFR knock out mean = 76.9 ± 6.3 pA; mean action potentials at two times rheobase in wild type mice = 2.1 ± 0.5 vs. TNFR knock out mean = 1.8 ± 0.5). After TNF-α incubation, the rheobase in the knockout mice DRGs was 52% higher (n = 17, p = 0.003), and the mean number of action potentials at twice rheobase was 44% lower (p = 0.04), than in wild-type (Figure 5-4A).

The effects of UC-supernatant on neuronal excitability (see Figure 5-1) were next examined in the TNF-α receptor knockout mice to assess what contribution TNF-α made to the overall supernatant effect (Figure 5-4B).
Figure 5-4 The effects of ulcerative colitis (UC) supernatant and tumor necrosis factor α exogenous (TNF-α) on neuronal excitability are markedly attenuated in TNF-α receptor knockout mice. Data shown illustrates reversal of effects of (A) UC-supernatant and (B) TNF-α on excitability of DRG neurons isolated from TNFR knockout (black bars) and wild-type mice (white bars). Data represents mean ± SEM. *P < 0.05. Numbers in parentheses indicate numbers of neurons tested.
The rheobase in neurons incubated in the UC-supernatant (n = 17) was 45% higher (Figure 5-4) than in wild-type neurons (n = 17; p = 0.05). The number of action potentials at twice rheobase was decreased by 46% compared to wild-type (p = 0.03).

**TNF-α and UC-supernatants modulates Kv and Naᵥ currents**

Voltage-gated Na+ and K+ channels are fundamental in determining neuronal excitability and, we and others have shown previously that inflammation can modulate these channels in DRG neurons (Beyak et al., 2004; Beyak & Vanner, 2005; Rush & Waxman, 2004; Tan et al., 2006). Acute application of TNF-α has been shown to modulate Nav 1.8 currents in somatic DRG neurons (Jin & Gereau, 2006), but the effect of overnight incubation of TNF-α and UC-supernatant on these and Kv currents is unknown.

Thus, to understand the ionic mechanisms underlying the TNF-α induced hyperexcitability of mouse nociceptive DRG neurons, we isolated voltage-gated potassium (Kv) currents based on their inactivation properties (Figure 5-5A) and compared current densities between control and those incubated in TNF-α (100 ng/ml) or UC-supernatant and control supernatant. TNF-α incubation caused a significant decrease of Iₐ (p = 0.01) and Iₖ (p = 0.0003) current densities respectively, compared to control neurons (Figure 5-5B). Similarly, UC-supernatant incubation caused a significant decrease of Iₐ (p = 0.007) and Iₖ (p = 0.006) current densities respectively, compared to control supernatant (Figure 5-
Figure 5-5 Tumor necrosis factor α (TNF-α) and ulcerative colitis (UC) supernatant suppresses Kv⁺ currents. (A) Representative voltage clamp trace of Kv⁺ currents isolated while holding at -100 mV (total currents) or -60 mV (Iₖ), using a 500 ms voltage step in 10 mV intervals. Subtraction of Iₖ from the total current yielded Iₐ [-100 - (-60)]. Arrow heads indicate the points at which current amplitudes were measured and analyzed for the corresponding voltage steps. Current –voltage relationship of the isolated Iₐ (left) and Iₖ (right) currents illustrate significant suppression of both currents in TNF-α (B) or UC-supernatant (C) treated neurons. (D) Effects of exogenous TNF-α (left) or UC-supernatant (right) on the steady-state inactivation properties of isolated Kv⁺ currents. The steady-state inactivation curves for both Iₐ (left) and Iₖ (right) currents in TNF-α or supernatant incubated neurons were obtained by plotting the normalized test current amplitudes against conditioning pre-pulse potential and were fitted using Boltzmann function. Incubation with TNF-α or UC-s resulted in a hyperpolarizing (left) shift of the steady-state inactivation curve for both currents. Data represents mean ± SEM. *P < 0.05, **P < 0.001, ***P < 0.0001, two-way ANOVA with Bonferroni post-test.
Incubation with TNF-α or UC-supernatant did not alter the voltage dependency of activation of either $I_A$ or $I_K$ currents (data not shown). However, there was a hyperpolarising shift of the steady-state inactivation curve for both currents with TNF-α and the $I_A$ current with the UC-supernatant. The $V_h$ for the steady-state inactivation of $I_A$ in TNF-α treated and control neurons were $-91.6 \pm 0.7$ mV and $-80.1 \pm 0.5$ mV $p = 0.0001$, with $k$ values of $-10.04 \pm 0.5$ and $-10.2 \pm 0.5$ respectively; whereas the $V_h$ of $I_K$ in TNF-α treated and control neurons were $-72.1 \pm 0.4$ mV and $-65.9 \pm 0.3$ mV $p = 0.001$, with $k$ values of $8.8 \pm 0.3$ and $9.6 \pm 0.3$ respectively. The $V_h$ for the steady state inactivation of $I_A$ in the UC-supernatant and control supernatant neurons were $-86.4 \pm 0.7$ mV and $-76.2 \pm 0.8$ mV, $p 0.001$; with $k$ values of $-12.3 \pm 0.5$ and $-11 \pm 0.7$ respectively.

$Na_v1.8$ currents were isolated by including tetrodotoxin (1 μM) in the bath solution to block TTX sensitive currents. As illustrated in Figure 5-6, overnight incubation of colonic DRG neurons with exogenous TNF-α ($n = 8$ cells for TNF-α and controls) or UC-supernatant ($n = 9$ for UC-supernatant and $n=10$ for control supernatant) enhanced the TTX-R $Na_v1.8$ currents. The current-voltage relationship demonstrated activation of the currents at $\sim -42$ mV, with a peak close to $-10$ mV ($p = 0.001$ for TNF-α and $p = 0.009$ for UC-supernatant). TNF-α and UC-supernatant did not affect either the voltage dependency of activation or steady state inactivation of the currents (data not shown).
Figure 5-6  Tumor necrosis factor α (TNF-α) and ulcerative colitis (UC) supernatant enhances tetrodotoxin resistant (TTX-R) Na\textsubscript{v} 1.8 currents. (A) Representative voltage clamp trace of TTX-R Na\textsubscript{v} 1.8 currents isolated using 1 μM TTX from control (left trace) and TNF-α (right trace) incubated neurons. Cells were held at a holding potential of -100 mV and stepped from -80 to +35 mV in 10 mV intervals for a duration of 200 ms. (B) Current – voltage relationship for isolated TTX-R Na\textsubscript{v} 1.8 currents showing significant augmentation of currents in neurons incubated in TNF-α (left) or UC-supernatant (right) as opposed to control media or C-supernatant, respectively. Data represents mean ± SEM. *P < 0.05, **P < 0.001, two-way ANOVA with Bonferroni post-test.
DISCUSSION

The inflammatory milieu surrounding the axon terminals of nociceptive DRG neurons in the intestine contains a wide repertoire of inflammatory mediators, many of which can either directly activate or sensitize these neurons (Bueno & Fioramonti, 2002; Khan et al., 2008; Obreja et al., 2005; Shanahan & Targan, 1992; Viviani et al., 2007) and, in some cases, have opposing anti-nociceptive actions (Auge et al., 2009; Beck & Wallace, 1997; Verma-Gandhu et al., 2007; Viviani et al., 2007). The exact nature of these mediators depends partly upon the type of inflammation (e.g. UC vs. Crohn's disease vs. infectious colitis). Given this variability, the first aim of this study was to determine the net effect of the inflammatory milieu on the excitability of nociceptive neurons innervating the inflamed region of the colon in UC. UC is a chronic inflammatory disease of the colonic mucosa and submucosa, and this inflamed tissue is readily accessible through pinch biopsies obtained during endoscopic procedures.

We incubated neurons in supernatant containing secreted inflammatory mediators from the mucosa and submucosa for ~ 24 hours to mimic the inflammatory milieu in human active UC (i.e. as opposed to acute application for minutes) and found that this supernatant induced hyperexcitability in colonic nociceptive neurons. Since enhanced neuronal excitability can mediate increased nociceptive signaling (Kayssi et al., 2007; Song et al., 2006), our results suggest that the inflammatory cytokine profile from human UC patients can play an important nociceptive role in these patients. The second aim of this
study was to identify a key mediator underlying this response. We examined whether TNF-α was important because it was known to directly activate DRG neurons, and clinical therapies currently exist to target its actions in humans.

Several lines of evidence suggested that TNF-α was central to the neuronal excitability changes evoked by the human supernatants in our study. We observed high levels of TNF-α in the supernatant from UC patients, and confirmed the presence of TNF-α receptor 1 transcript on labeled DRG neurons (Hensellek et al., 2007; Inglis et al., 2005; Li et al., 2004; Pollock et al., 2002; Shubayev & Myers, 2001). To ensure colonic projecting neurons were used in the RT-PCR studies, we used laser capture microdissection to isolate Fast Blue labeled DRG neurons. Previous studies (Jin & Gereau, 2006) of unidentified DRG neurons demonstrated that acute TNF-α application could modulate Na\(_{v}\) channels in DRG neurons, but the effects on neuronal excitability and colonic neurons per se, were unknown. Although we did not directly compare supernatant and TNF-α effects, we found that incubation of TNF-α (24 hrs) for the same duration resulted in very similar actions on neuronal excitability, i.e. decreased rheobase and increased action potential discharge compared to controls. Moreover, when these findings were examined in TNFR knockout and wild-type mice, the decrease in supernatant and TNF-α effects were similar in knockout animals. Together, these findings suggest that abrogation of the TNF-α effect on nociceptive DRG neurons could have significant effects on pain in these IBD patients. Changes in rheobase and rate of action potential discharge, as
seen in the present study following supernatant and TNF-α incubation, can result from modulation of one or more of the voltage-gated ion channels underlying action potential generation.

In TNBS colitis and ileitis animal models of IBD, neuronal hyperexcitability is associated with modulation of both $K_v$ and $Na_v$ 1.8 currents (Beyak et al., 2004; Beyak & Vanner, 2005; King et al., 2009; Stewart et al., 2003). There is also evidence that acute application of individual inflammatory mediators, including TNF-α can modulate one or more of these channels in a similar fashion (Kayssi et al., 2007; Khan et al., 2008; Obreja et al., 2005; Rush & Waxman, 2004; Song et al., 2006). We tested the effects of TNF-α incubation and the UC-supernatant using voltage clamp techniques and found significant reductions in transient $I_A$ and delayed rectifier $I_K$ currents and enhancement of the $Na_v$ 1.8 currents, both of which would lead to enhanced neuronal excitability. The intracellular signaling event(s) which mediate the TNFR responses remain to be fully elucidated but post-translational and transcriptional changes in these ion channels have been implicated (Qian et al., 2009; Sergeant et al., 2005). We observed a leftward shift in the $I_A$ inactivation curve suggesting a smaller proportion of available channels could be activated at a given voltage, and as a result could contribute significantly to the enhanced neuronal excitability observed in our study. This finding has been observed in a number of inflammatory models (Beyak & Vanner, 2005; Ibeakanma et al., 2009; Qian et al., 2009; Stewart et al., 2003) and although the mechanism underlying this shift is unclear, post-translational
modification, such as phosphorylation of these channels through protein kinases has been proposed (Qian et al., 2009; Sergeant et al., 2005). Our previous molecular studies of the expression of Na\textsubscript{v} 1.8 channels during inflammation also suggested post-translational mechanisms may underlie the observed increases in Na\textsubscript{v} 1.8 currents and we failed to demonstrate increased Na\textsubscript{v}1.8 transcript following 24 hr TNF-\(\alpha\) incubation (King et al., 2009).

Together, these results suggest that post-translation modulation of channels are important, although they do not exclude that transcriptional changes may also occur during the evolution of inflammatory processes (Beyak & Vanner, 2005; King et al., 2009; Qian et al., 2009). Previous studies have implicated both p38 and ERK signaling in such events (Hensellek et al., 2007; Jin & Gereau, 2006) but detailed studies with selective antagonists of these intracellular pathways are needed to determine whether one or more of these pathways preferentially modulates specific ion channels.

In summary, we have shown that supernatants obtained from mucosal biopsies from patients with UC induced hyperexcitability of nociceptive colonic DRG neurons. Furthermore, we have found that TNF-\(\alpha\) is a major mediator of these events and that modulation of multiple ion channels are involved. Thus, the treatment of UC patients with anti-TNF-\(\alpha\) therapy such as infliximab could decrease pain, at least in part, by directly inhibiting the action of TNF-\(\alpha\) on nociceptive neurons. It is possible that other mediators in the supernatant have acute actions on neuronal excitability which desensitized during the more
sustained exposure examined in our study. Although we studied changes in ion channels in the soma to reflect what occurs in the nerve terminal within the inflamed intestine, there may be additional mechanisms restricted to the nerve terminal, such as immune secretion of anti-nociceptive factors (Auge et al., 2009; Verma-Gandhu et al., 2007) which could further modulate the changes we observed. This study focused on patients with UC given the relative ease of access to inflamed tissue in the distal colon, however given the evidence that the balance of pro- and anti-nociceptive factors may differ in patients depending on the nature of the pathophysiology of the inflammation (Auge et al., 2009; Verma-Gandhu et al., 2007), it will be important to determine whether important differences exist in patients with Crohn’s disease or other pathology such as infectious or ischemic colitis. It will also be important to dissect the contribution of other pro-nociceptive factors, including those released by bacteria e.g. LPS and lipoproteins, to determine their direct and indirect contribution to nociception in these patients.
CHAPTER 6: GENERAL DISCUSSION
The main objective of the studies described in this thesis was to increase our understanding of the peripheral mechanisms underlying inflammation-induced nociceptive signaling in animal models and patients with IBS and IBD.

The key findings of this thesis were:

1) Acute colitis resulting from *C. rodentium* infection induces sustained abnormal nociceptive DRG signaling.

2) Proteases are key peripherally secreted factors mediating post-infectious *C. rodentium*-induced abnormal nociceptive DRG signaling.

3) The interaction of stress hormones and local inflammatory mediators in colonic tissues can have a synergistic action which increases peripheral nociceptive DRG signaling in PI-IBS.

4) TNF-α is a key mediator underlying enhanced nociceptive DRG signaling in human ulcerative colitis.

5) Inflammation-induced hyperexcitability of the colonic DRG neurons in IBS and IBD involves modulation of specific Na\textsubscript{v} and K\textsubscript{v} voltage gated ionic currents.

The following section examines potential limitations of the techniques employed in these studies. Subsequent sections address broader implications of the findings, which were not discussed in detail in the relevant chapters.
IMPLICATIONS OF TECHNIQUES AND MODELS EMPLOYED IN THIS THESIS

A major strength of the studies described in this thesis is that excitability of colonic DRG neurons was assessed using a number of different experimental techniques. Each technique has unique advantages and disadvantages, but together they complement each other when combined. The merits of each of these techniques are discussed below. Another strength of these studies was the use of supernatants obtained from human colon biopsies which increased the translational capacity for the studies. The value and limitations of this approach are also discussed in a subsequent section.

Patch clamp recording from isolated cell bodies: This approach is widely adopted by many investigators to evaluate neuronal electrical events using either whole cell or perforated patch configurations (in current- or voltage-clamp modes [Gold et al., 1996; Lomax et al., 2007]). This technique provides information regarding activities of ion channels that underlie neuronal excitability and, enables assessment of responses of the respective neurons to inflammatory mediators or stress hormones. However, a number of issues regarding the use of the technique remain unresolved. For example, it is not clear whether recordings obtained from an isolated soma precisely reflect the electrical events occurring at the nerve terminals in situ. Nonetheless, a number of recent studies have provided evidence that seem to support the notion that information obtained from cell bodies of sensory neurons is, at least in part, reflective of events occurring at
nerve endings. For example, application of protease activated receptor 2 (PAR$_2$) agonist (SLIGRL) increased jejunal afferent nerve firing (mesenteric nerve terminals) in one study; and in another independent study induced hyperexcitability of colonic DRG neurons (Kayssi et al., 2007; Kirkup et al., 2003), suggesting the receptor is present in both the soma and the peripheral terminals.

Another issue results from the need to isolate and culture neurons, which disrupts connections the axons make with other cell types (e.g. glia) that surround them in their natural environment, and with central facilitatory and/or inhibitory pathways thought to influence pain perception and expression. As a consequence, recordings from such dispersed cells could potentially provide misleading information regarding their behavior. In addition, the isolation and culture processes may lead to changes in the phenotype of the cells which in turn could give rise to exaggerated or reduced responses when recordings are obtained from such cells. Furthermore, in their natural environment (whether in a healthy or diseased state) afferent nerve terminals are continuously exposed to a wide range of mediators (both pro- and anti-inflammatory). These latter two concerns, however, are mitigated by making comparisons with control neurons which were isolated in identical fashion. Finally, in vitro patch clamp studies of isolated neurons are usually performed at temperatures (~ 22 – 23 °C) different from that of their natural environment, which would also not reflect changes in temperature due to tissue inflammation. Despite these issues, patch clamp techniques have emerged as very valuable tools in the study of excitable cells.
because the technique can provide valuable information regarding neuronal excitability and the properties of ion channels which underlie these changes.

**Multi-unit afferent nerve fiber recording:** The use of multi-unit afferent recording is one means of addressing some of the concerns highlighted above because it provides a measure of changes in excitability at the nerve terminal-tissue interface and electrical events downstream from the site of axotomy (Keating *et al.*, 2008; Larsson *et al.*, 2006). This technique allows for testing of responses to physiological stimuli such as distention of the colon and to examine the response to inflammatory mediators. Technically, the method requires that a minimum number of units be simultaneously recorded from to reduce the summation of action potentials, otherwise discharge patterns (i.e. amplitude and waveforms) of the individual units will be difficult to interpret. Recording from single units provides additional information including the specific involvement of low and high threshold units (Yu *et al.*, 2008), but is technically much more difficult to perform. A major disadvantage of afferent nerve fiber recording technique is that it does not provide direct information regarding activities of ion channels (which are responsible for initiating action potentials) present on the nerve terminals. However, as described above, the combined use of patch clamp studies can compensate for these disadvantages.

**Visceromotor reflexes:** Visceromotor reflexes complement multi-unit afferent recording by providing a measure of nociception in conscious animals. With this approach, changes in sensitivities of hollow organs of the viscera after
inflammation or irritation are evaluated in conscious animals, including humans, using distension apparatus such as balloons (Christianson & Gebhart, 2007). The distensions are mechanical stimuli that aim to produce adequate stimuli for pain elicitation (intensity) and visceral pain referral patterns. In animal studies, colorectal distension (CRD) has been widely and reliably employed as a model (Cenac et al., 2007; Christianson & Gebhart, 2007). The limitation of this approach is that the interpretation of the results are more complex because the observed changes could be due to changes at the nerve terminal tissue interface, the intrinsic excitability of the nerves or at the level of the spinal cord where complex integration may occur, including descending inhibitory responses. Another concern expressed by investigators is that the method is technically challenging in order to obtain consistently reproducible results.

**Human biopsies as models for the study of disease mechanisms:**
The use of human biopsies to study disease mechanisms, particularly in IBS, has helped to increase translational relevance of findings (Barbara et al., 2007; Cenac et al., 2007). These studies have been particularly important because they have demonstrated that immune activation and specific mediators, such as histamine, trypsin, tryptase, and serotonin, exist in mucosal tissues from IBS patients (Barbara et al., 2007; Cenac et al., 2007; Klooker et al., 2010), substantiating the claim that peripheral events contribute to the pathogenesis of IBS, at least in many patients (Anand et al., 2007; Costigan & Woolf, 2000). This approach also enables examination of combined effects of representative mediators (both pro-
and anti-inflammatory) present in the tissue samples. In our studies for instance, this technique enabled us to demonstrate that mucosal biopsies from IBS and IBD patients express key inflammatory mediators (proteases and TNF-α respectively), and that supernatants produced from these tissues induces abnormal DRG neuronal signaling. One potential limitation of this approach is that it is implicit that the colonic nociceptors would be modulated by the inflammatory milieu expressed in the biopsies. However, mucosal fibers may not be nociceptors based on research studies (Beyak et al., 2006) and practical experience. For example, it has been observed that patients undergoing mucosal biopsy sampling do not feel the pain. It is possible that the mediators in the mucosal biopsies are representative of those in deeper tissues where nociceptors are present. Alternatively, or in addition, it is possible that axon collaterals of mucosal fibers are nociceptors (e.g. those associated with submucosal arterioles and that sensitization of both of these nerve terminals occurs. Further studies are needed to clarify this issue.

Another potential limitation of this model is that tissue processing and storage could lead to degradation of the mediators and this could lead to misleading results when used for studies. Also, subtle differences that might impact adversely on experimental results may exist due to the nature of the biopsies and the regions (i.e. whether from the left or right side) of the gut where biopsy samples are taken.
Peripheral mechanisms play a critical role in abnormal nociceptive signaling in PI-IBS, where symptoms persist following resolution of inflammation

Visceral sensory dysfunction (hypersensitivity), a common finding in patients with IBS, was first demonstrated in patients who were subjected to colonic balloon distension by Ritchie in 1973, and was later confirmed in other studies (Ritchie, 1973; Whitehead et al., 1990). Typically, IBS patients report pain in response to stimuli that would not be perceived by healthy controls (allodynia) and exaggerated pain in response to noxious stimuli (hyperalgesia) (Ritchie, 1973; Whitehead et al., 1990). However, the mechanisms underlying visceral hypersensitivity in IBS are not clear. While some studies identified important associations relating to central nervous system signaling mechanisms (e.g. alterations by anxiety, hypervigilance, depression, abnormal central sensory processing) (Anand et al., 2007; Woolf, 1995), others implicated abnormal peripheral sensory DRG signaling mechanisms (e.g. alterations by immune signaling including mast cells) (Anand et al., 2007; Costigan & Woolf, 2000).

The findings of this thesis provide further evidence for the importance of peripheral mechanisms and novel insights into some of the pathways. For instance, we found that C. rodentium infection in mice established colitis with important pathophysiological features consistent with human studies of PI-IBS
(Anand et al., 2007; Barbara et al., 2002; Barbara et al., 2004b; Costigan & Woolf, 2000; De & Barbara, 2008). This self-limiting colitis caused excitation of nociceptive DRG neurons which persisted following resolution of infection and features of inflammation, and increased afferent nerve discharges in response to colon distension. These findings parallels human studies that suggest IBS pain symptoms result from alterations of afferent neuron signaling from the gut (Barbara et al., 2007; Whitehead & Crowell, 1991). Together these observations further establish a role for peripheral mechanisms in abnormal nociceptive signaling in PI-IBS.

There are several features of the *C. rodentium* model employed in our studies which may allow for more accurate study of mechanisms in PI-IBS. Unlike many other animal models (Krauter et al., 2007; Lomax et al., 2007), the sustained abnormal sensory signaling observed with our *C. rodentium* model occurred in the absence of structural mucosal abnormalities or overt inflammation, as seen in human IBS (Barbara et al., 2002; Barbara et al., 2004b; De & Barbara, 2008). In these other studies, colitis was induced using agents (chemical or parasitic) that produced severe mucosal inflammation and tissue damage not found in human IBS. Furthermore, *C. rodentium* bacteria share similar virulence factors with human *E. coli*, and as such attach to epithelial surfaces to cause ultra-structural changes known as effacing and attaching lesions in a characteristically similar fashion as *E. coli* (Luperchio & Schauer, 2001; Mundy et al., 2005). This bacteria is commonly implicated in human PI-IBS.
We also found that tissue protease levels were increased following the infection in this model, another common finding in IBS (Cenac et al., 2007; Scully et al., 2010). Thus, the properties of our post-infectious C. rodentium animal model suggest that it may enable the detailed examination of mechanisms of a number of key features of human IBS.

Proteases are key peripherally secreted factors contributing to abnormal nociceptive signaling in PI-IBS

Based on studies in human, tissue proteases are among the important secreted mediators reportedly found in inflamed tissue environments of IBS patients (Cenac et al., 2007; Scully et al., 2010). Among the different classes, serine proteases have particularly been suggested to be important candidates for pain expression and the development of visceral hypersensitivity (Cenac et al., 2007; Gecse et al., 2008). Our findings using the post-infected C. rodentium model parallels findings of these human studies. For example, we found elevated levels of serine proteases in post-infected C. rodentium colonic tissue samples (see chapter 3). We also found that trypsin, a representative member of the serine protease subfamily alters excitability of colonic DRG neurons. Together, the findings in human IBS studies and our post-infected C. rodentium model demonstrate that proteases are key peripherally secreted factors contributing to abnormal nociceptive signaling in PI-IBS. The mechanisms underlying this altered neuronal signaling are not completely understood, but a number of
studies have shown that serine proteases can signal through cleavage of protease activated receptor 2 (PAR₂), a member of the G-protein coupled receptor expressed on nociceptors (Kayssi et al., 2007).

Whether cysteine proteases also make a contribution to visceral pain expression (through direct activation of visceral nociceptive DRG neurons) and the development of visceral hypersensitivity has yet to be clarified. A recent study suggested that cathepsin-s (Cat-S), an important member of cysteine proteases, can participate in neuropathic pain expression (Barclay et al., 2007; Clark et al., 2007), and that it is capable of cleaving PARs (Reddy et al., 2010) which are readily expressed on visceral nociceptive neurons, suggests Cat-S could be important in visceral pain expression in IBS. Indeed, we found that Cat-S altered properties of colonic nociceptive neurons and provoked visceral hyperalgesia to graded colonic distensions in mice. The relevance in humans of these novel animal findings was suggested by the observation that a specific cysteine protease inhibitor (E-64) inhibited neuronal hyperexcitability evoked by human IBS-supernatants. Together these findings suggest that cysteine proteases could be important mediators of the neuromodulatory actions of human IBS-supernatants and contribute to visceral pain signaling.
Central and local gut factors interact to increase peripheral sensory DRG signaling in PI-IBS

Psychological stressors have been implicated as possible factors associated with alterations of afferent neuron functions and pathways of visceral sensory transmission in IBS (Barreau et al., 2008; De & Barbara, 2008; Mayer & Gebhart, 1994; Santos et al., 2008), but the mechanisms are not fully understood. Our studies described in chapter 3 of this thesis show that combining repeated WAS and self-limiting C. rodentium infection exaggerated peripheral DRG signaling and enhanced visceral hyperalgesia and allodynia, through interactions between proteases (local factors) and stress hormones (central factors).

One challenge was to determine whether the stress hormones exerted indirect effects by increasing pro-nociceptive mediators in the colonic tissues to increase peripheral nociceptive signaling or acted directly through activation of receptors on the DRG neurons. Previously, it has been suggested that chronic stress acted by increasing peripherally circulating corticotropin releasing factor (CRF) which activates mucosal mast cells, causes release of proteases (Santos et al., 2008; Vicario et al., 2010). These proteases, in turn, can activate PAR2 expressed on colonocytes to alter mucosal permeability and immune signaling in the colon (Jacob et al., 2005). However, when tested in our system, we found no evidence of WAS induced alteration of mucosal structure or immune activation, suggesting that the stress hormones can signal directly to the neurons. Therefore, a conceptual hypothesis for the interaction observed in our study is
that stress hormones, e.g. corticosterone and epinephrine, and inflammatory mediators, e.g. proteases, converge on nociceptive DRG neurons to increase neuronal excitability through synergistic actions.

We tested this hypothesis in \textit{in vitro} studies by first showing that exogenous application of stress hormones could recapitulate the effects of chronic stress on neuronal excitability. We then showed that a sub-threshold level of PAR$_2$-AP (a synthetic analogue of tethered ligand of the receptor that activates PAR$_2$ receptors) that had no effect on neurons from unstressed mice produced enhanced neuronal excitability when combined with stress. It is conceivable that stress released hormones (i.e. epinephrine and corticosterone) sensitized afferent nerve terminals which then were driven to threshold levels of excitation by the sub-threshold level of PAR$_2$-AP. The mechanism which underlies this interaction is unclear, but could result from stress-induced up-regulation of expression of PAR$_2$ in the cell membrane (Kayssi \textit{et al.}, 2007), and/or enhancement of intracellular signaling pathways linking PAR$_2$ to ion channel targets (Kayssi \textit{et al.}, 2007), and/or modulation of downstream ion channels underlying action potential electrogenesis.

Together, our findings may be clinically relevant in that repeated stress may sustain PI-IBS symptoms by facilitating peripheral signaling pathways that otherwise would not be detectable. Furthermore, these observations may provide a unifying mechanism to explain stress-induced increases in sensory signaling in other generalized pain conditions such as fibromyalgia or interstitial cystitis.
(Okragly AJ et al, 1999). It is well known that these conditions are characterized by varying degrees of inflammation and altered immune functions, and most often associated with stress-related disorders such as depression and anxiety (Buskila & Cohen, 2007). Thus, in likewise manner, afferent sensitization resulting from repeated stress hormones could predispose the nerve terminals to a state of constant hyper-responsiveness to stimulation by sub-threshold level of secreted mediators to sustain nociceptive signaling.

**TNF-α is an important mediator of the neuromodulatory actions of human ulcerative colitis supernatant on nociceptive DRG neurons innervating the colon**

TNF-α is a multifunctional cytokine produced mainly by activated monocytes, macrophages and natural killer cells. Cellular effects of TNF-α are mediated through binding to either of its specific cell surface receptors, TNFR1 or TNFR2. TNFR1 is present in most tissues, whereas TNFR2 is primarily expressed in immune and endothelial cells (Locksley et al., 2001).

In recent somatic animal studies, it was shown that TNF-α activated afferent C-fibers and, induced mechanical allodynia and hyperalgesia when subcutaneously or perineurially applied (Junger & Sorkin, 2000; Reeve et al., 2000). Also, in an animal model of neuropathic pain, a TNF-α neutralizing agent attenuated thermal hyperalgesia and mechanical allodynia (Sommer et al., 2001). These somatic effects suggest TNF-α can directly activate C-nociceptors, but its effects on
visceral afferent neurons and whether it contributes to pain expression in IBD was yet to be shown.

Our studies show for the first time that laser-captured Fast-Blue labeled colonic DRG neurons express TNFR1, and that TNF-α plays a major role in inducing the excitatory effects of human UC supernatants on colonic DRG neurons (see chapter 5). These effects were not observed in neurons from TNFR knockout mice, suggesting the TNF-α is a key mediator in the inflammatory cytokine profile of human UC supernatant. As a result, treatments (e.g. inflixamab) directed at reducing TNF-α levels should also decrease peripheral pain signaling in these patients.

It was not a goal of this thesis to directly compare nociceptive signaling in IBS and IBD or effects of inflammatory mediators but our studies show that TNF-α may be particularly important in IBD while protease may be key to nociceptive signaling in IBS. This however does not exclude a role for TNF-α or proteases in nociceptive signaling in either or both of the disorders, and although we did not examine actions of other cytokines in these studies, other studies suggest several inflammatory mediators can directly sensitize nociceptive afferent neurons (Gold et al., 1996). For example, PGE2 has been shown to induce sensitization of cultured rat sensory DRG neurons through a $\mathrm{Ca}_{2}\!^+$ dependent mechanism (Gold et al., 1996). Thus, it will be interesting to know how these other mediators may impact visceral nociceptive signaling.
Specific secreted mediators provoke inflammation-induced changes in peripheral sensory DRG signaling through modulation of specific ionic currents

Numerous studies show that specific ion channels are modulated by inflammation to induced DRG neuronal hyperexcitability (Beyak et al., 2004; Beyak & Vanner, 2005; Kayssi et al., 2007). Given evidence that voltage gated sodium (Na\(_\text{v}^+\)) and potassium (K\(_\text{v}^+\)) currents regulate neuronal excitability through changes in activation threshold, action potential electrogenesis, and spike frequency (Cervero & Laird, 2004; Rush et al., 1998), and that these channels are abundantly expressed in nociceptive DRG neurons (Akopian et al., 1996; Djouhri et al., 2003; Yoshimura et al., 1996), we examined their roles in altered nociceptive DRG signaling in IBS and IBD using our post-infectious and human biopsy supernatant models. We found (see chapters 2 and 5) that visceral inflammation and TNF-α significantly decreased transient I\(_A\) and delayed rectifier I\(_K\) current densities, and enhanced Na\(_\text{v}\) 1.8 currents, all of which would lead to enhanced neuronal excitability and hence increased pain signaling. Thus, it appears that regulation of multiple voltage gated ion channels occur during inflammation. We did not study other ion channels such as the transient receptor potential (TRP) and K\(_2\)P channels, but previous studies have shown that they also can be modulated by inflammation to increase nociceptive signaling (Caterina et al., 2000; Davis et al., 2000). For example, deletion of TRPV1 channel in mice results to impairment of inflammation-induced pain sensation.
and development of thermal hyperalgesia (Caterina et al., 2000; Davis et al., 2000). Taken together, our findings and related studies suggest there are multiple ion channel targets underlying the increased neuronal excitability and mechanosensitivity observed in our studies.

Changes in current densities of these channels could arise from increased or decreased synthesis of new channel proteins (i.e. transcriptional changes); or from modulation of existing ones in the cell membrane (i.e. translational / post-translational changes). At the moment, we do not know whether the altered Na$_v$ and K$_v$ current densities observed in our studies are due to translational and/or post-translational events (phosphorylation, dephosphorylation, and/or transportation / insertion of existing channels into the cell membrane) or due to transcriptional production of more channels. Previous studies that examined the role of these cellular processes in inflammation-induced changes in sensory signaling are conflicted (Kings et al., 2009; Tanaka et al., 1998). For example, a recent study found increased Na$_v$ 1.8 currents in DRG neurons innervating inflamed colon (TNBS colitis) associated with increased protein expression but not the messenger transcript, suggesting translational / post-translational mechanisms (King et al., 2009). Others observed increased Na$_v$ 1.8 mRNA transcripts associated with increased protein levels in a carageenan-induced paw inflammation, suggesting involvement of transcriptional events (Tanaka et al., 1998). Therefore, detailed molecular and pharmacological studies of these
intracellular pathways are needed to determine whether one or more of these pathways underlie the altered $Na_v^+$ and $K_v^+$ current densities.

CONCLUSION

In conclusion, this thesis has identified a number of important molecular targets contributing to inflammation-induced changes in peripheral DRG signaling. The mechanisms involve release of important mediators like TNF-α (IBD), serine and cysteine proteases (IBS) which directly or indirectly modulate voltage gated ion channels expressed on colonic DRG neurons thereby altering their intrinsic excitability. Moreover, effects of these inflammatory mediators on DRG neuronal excitability are markedly increased by chronic stress through actions of stress mediators. Therefore therapeutic interventions targeting pathways specific to one or more of these molecules could prove helpful in the management of pain symptoms in IBD and/or IBS. The molecules identified in our studies appear to play a dominant role, making them appealing targets for treatments. However, further studies are needed to determine if other mediators could also be important, particularly in human tissues.

FUTURE DIRECTIONS

In recent years, there has been increasing interest in luminal microbiota as potential aetiopathogenic factors for the development and maintenance of symptoms in both IBS and IBD. (Furrie et al., 2005; Hoveyda et al., 2009). For
example, we and others have shown that acute bacterial gut infection induces sustained sensory dysfunctions in the gut (Bercik et al., 2004; Ibeakanma et al., 2009). Furthermore, other studies suggested that quantitative or qualitative differences exist in the microbiome of IBS patients (Lee & Tack, 2010), and that resulting differences in signaling to the colonic effector cells (e.g. epithelium, innate immune cells) from this microbiota could result in symptoms. This reasoning is supported by specific probiotic therapy which was shown to partially reverse the gut dysfunction in IBS (Hoveyda et al., 2009) and IBD (Furrie et al., 2005). However these observations raise a number of questions such as: How do the microbiota and host communicate to increase pain symptoms in these conditions and what are the underlying mechanisms of the interactions? A recent study reported a 5-fold increase in toll-like receptor 4 (TLR4) in women with IBS over healthy controls (Brint et al., 2011). In addition, we recently showed that DRG neurons expressed TLRs (Ochoa-Cortes et al., 2010), raising the possibility that the microbiome, at least in certain settings, might signal directly to neurons. To support this notion, we found that the TLR4 ligand, lipopolysaccharide (LPS; a bacterial cell wall product), alters the excitability of colonic DRG neurons, and increases transcripts of important pro-inflammatory cytokines thorough NF-κB and an as yet unidentified signaling pathway (Ochoa-Cortes et al., 2010). Clarifying these pathways and the possible contribution of other TLRs are needed because these potentially could provide novel visceral pain treatment paradigms.
Another mechanism by which microbiota could play a role in IBS is by altering intestinal permeability. Acute bacterial infection has been shown to trigger increased mucosal permeability mainly through disruption of epithelial tight junctions (Berkes et al., 2003). Failure of recovery of the disrupted tight junction was proposed as a possible mechanism underlying the sustained gut dysfunction, through constant immune activation by luminal bacteria (Barbara, 2006; Berkes et al., 2003). However, a clear knowledge of the underlying mechanisms and how the process may translate into increased sensory signaling remain lacking and require further investigation.
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Supplementary figure 3-1 Markers of colonic inflammation do not differ between *C. rodentium* (black bars) and *C. rodentium* plus WAS (white bars) animals. Data summarizing histopathology inflammation scores (total damage scores, inflammation, crypt epithelial proliferation/hyperplasia, recent and chronic damages) for colonic tissues from each group. Data are reported as mean ± SEM.
Supplementary figure 3-2 The expression of the occludin tight junction protein was not decreased in the colons of *C. rodentium* plus WAS animals compared to controls. (A) Western blot of tight junction protein occludin in four groups of animals; controls (saline gavage), WAS and saline gavage, *C. rodentium*, and *C. rodentium* and WAS. The three bands for each group represent protein obtained from colons in a separate group of animals. (B) Graph summarizing the mean optical densities of the bands in the western blot gel for each group. Data are expressed as the mean ± SEM of the three bands for each group (as shown in parentheses).
Supplementary figure 3-3 Serine but not cysteine protease activities are increased in post-infected colonic tissues. Representative gel micrographs illustrating serine (left; ~29 kDa mass) and cysteine protease (right) activities in control and C. rodentium infected mouse colonic tissues. Activities were detected using small molecule inhibitor-base (activity-based) probes that covalently interact with the activated enzymes. β-actin was used as a reference protein to ensure equal amounts of proteins are loaded in the gels.
PUBLICATIONS

**Manuscripts**


**Charles Ibeakanma**, Fernando Ochoa-Cortesa, Marcella Miranda-Morales, Todd MacDonald, Ian Spreadbury, Nicholas Cenac, Fiore Cattaruzza, David Hurlbut, Stephanie Vanner, Nigel Bunnett, Nathalie Vergnolle, Stephen Vanner. Mediators of Brain-Gut Interactions Increase Peripheral Nociceptive Signaling in a Post-Infectious IBS Model (Gastroenterology; accepted for publication with minor revisions).

Fiore Cattaruzza, Victoria Lyo, Ella Jones, David Pham, Kimberley Kirkwood, James Hawkins, Eduardo Valdez-Morales, **Charles Ibeakanma**, Stephen Vanner, Mathew Bogyo, Nigel Bunnett. Cathepsin S is Activated During Colitis and Causes Visceral Hyperalgesia by a PAR2-dependent Mechanism in Mice (Gastroenterology; accepted for publication with minor revisions).

**Abstracts**


**CO Ibeakanma**, F Bautista-Cruz, M Richards, D Hurlbut, N Martin, S Vanner (2008) Sustained Hyperexcitability of Nociceptive DRG Neurons Following Inflammation in a *Citrobacter Rodentium* Model of Post-Infectious Irritable Bowel


