HOST STRESS AND THE GASTROINTESTINAL MICROBIOTA

THE INFLUENCE OF HOST STRESS ON THE GASTROINTESTINAL TRACT AND THE MICROBIOTA

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Abstract

Stress is known to play an important role in the natural history of gastrointestinal diseases, and functional disorders in particular. In health, activation of the stress response serves to maintain homeostasis in response to harmful stimuli. However, prolonged activation of the stress response can become maladaptive and contribute to the initiation and maintenance of symptoms in disorders such as irritable bowel syndrome (IBS). The mechanisms underlying this detrimental effect are unclear. This thesis investigates this relationship by examining the influence of 10 days of water avoidance stress on a murine model of acute bacterial gastroenteritis; a known trigger in a subset of IBS patients. Results indicate that stress can increase the level of the stress hormone norepinephrine in the gut. However, the overall influence of host stress during infection proves to be beneficial in this model, with decreased colonic inflammation and earlier clearance of the pathogen. Next, we utilized the olfactory bulbectomy (OBx) model of depression comorbid anxiety, which shows a heightened stress response, to examine mechanism underlying stress-mediated susceptibility in a more chronic setting. OBx resulted in increased neural activity and motility in the gut, and a change in composition of gut microbiota. These responses were not accompanied by changes in gut permeability or immune activation. Thus stress alters the habitat of commensal bacteria via a neurally mediated change in colonic motility. These results have bearing on the ability of stress to alter the microbiota: a feature of functional GI disorders.

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List of all Abbreviations

5-hydroxytryptamine	5-HT
Acetylcholine	ACh
Adrenocorticotropic hormone	ACTH
Anxiety and depression	AD
Attaching and effacing	AE
Autonomic nervous system	ANS
Central nervous system	CNS
Chronic life stress	CLS
Corticosterone	CORT
Corticotropin-releasing hormone	CRH
Denaturing gradient gel electrophoresis	DGGE
Enzyme linked immunosorbant assay	ELISA
Fluorescent-activated cell sorting	FACS
Food and water deprivation	FWD
Conductance	G
Glucocorticoids	GC
Gastrointestinal	G
Glucocorticoid receptor	GR
Health related quality of life	HRQOL
Healthy controls	HC
Hypothalamic-pituitary-adrenal	HPA
Short-circuit current	Isc
Interleukin-6	IL-6
Irritable bowel syndrome	IBS
Irritable bowel syndrome-constipation predominant	IBS-C
Irritable bowel syndrome-diarrhea predominant	IBS-D
Interdigestive motor complex	IMC
Locus ceruleus/norepinephrine	LC/NE
Lipopolysaccharide	LPS
Magnetic resonance imaging	MRI
Maternal separation	MS
Quantity	n
Olfactory bulbs	OB
Olfactory bulbectomy	OBx
Open field	OF
Potential difference	PD
Post-infectious irritable bowel syndrome	PI-IBS
Pro-opiomelanocortin	POMC
Paraventricular nucleus	PVN
Quality of life	QOL
Quorum sensing	QS
Step-down	SD
Small intestinal bacterial overgrowth	SIBO
Tail-suspension test	TST
Water avoidance stress	WAS

Declaration of Academic Achievement

I have completed the majority of the work contained within this thesis. I received technical assistance from the following individuals: Josh Collins (c-fos immunohistochemistry), Jennifer Jury (mounting tissues and running experiments for Ussing Chamber studies), Dr. Markus Geuking (FACS assay), Jun Lu (running the FACSArray Bioanalyzer System), and Patricia Blennerhassett (preparing mice for surgery, aiding in tissue preparation for contractility experiments).

Background

B.1. Irritable bowel syndrome (IBS)

B. 1. i. Overview of IBS

Irritable bowel syndrome (IBS) is a common and costly condition in our society [1] that is typified by altered gastrointestinal function with no associated underlying structural changes. Both the clinical presentation and the pathogenesis of this disorder are heterogeneous in nature, and the complex amalgam of influences that lead to its expression are only beginning to be understood.

Symptoms of IBS include abdominal pain, bloating and altered bowel habits. Patients may present with some or all of these symptoms in varying degrees. For example, patterns of bowel habit can separate IBS patients into subgroups characterized as being constipation predominant (IBS-C), diarrhoea predominant (IBS-D) or mixed presentation. Accordingly, effective treatment and underlying etiology of various subtypes may vary significantly. This complexity is reflected by a lower health related quality of life (HRQOL) compared to many other chronic diseases, and consumption of health care resources that is over 50% greater than matched controls [2]. Demographic risk factors include female gender, age, and socioeconomic status [2].

Other factors have been associated with IBS including genetic susceptibility, acute gastroenteritis, and stress. It is likely that none of these factors on their own will produce IBS, bur rather, that their combined influence results in the expression of disease.

The role of genetics in IBS has been considered due to the increased prevalence of IBS in relatives of patients. In a family case-control study that directly surveyed symptoms, Saito *et al.*, reported that 25% of relatives of IBS patients had IBS, compared to 12% of relatives of a non-IBS control group [3]. Additionally, some symptoms are more prevalent in patients with a family history of IBS, including loose stools, urgency constipation, and pain, suggesting that specific pathophysiological aspects or subgroups may have a genetic basis [4]. As well, the majority of twin studies report higher rates in monozygotic twins (i.e. genetically identical) than dizygotic twins; however, comparisons of twins reared together vs. apart have yet to be completed [4]. Genes that have been examined for their role in IBS generally fall into groups related to serotonin, adrenergic, inflammation, and intestinal barrier function [4]. While an extensive review is beyond the scope of this thesis, some notable associations include: serotonin receptor type 3 subunit gene HTR3E in two separate cohorts of female IBS-D [5]; genes encoding toll-like receptor 9, cadherin (a tight junction protein), and interleukin-6 (IL-6) in PI-IBS [6]; and a catechol-O-methyltransferase polymorphism (enzyme that degrades catecholamines) that was associated with increased bowel frequency [7]. Thus, genetics may play a role in IBS development and symptom expression; however, consistent reports of specific genetic variations are essential to understanding and exploiting the potential connection.

Acute gastroenteritis is a recognized risk factor for the development of IBS. Various studies indicate that seven to thirty-one percent of individuals who suffer from acute bacterial gastroenteritis develop IBS [Post-Infectious IBS (PI-IBS)] [8], and that additional host factors can increase the likelihood of such an occurrence. A prospective

study by Gwee *et al.* indicated that pre-existing psychological co-morbidities such as anxiety, depression, somatisation, and neurotic trait were strong predictors of PI-IBS development following gastroenteritis [9]. Spencer and Moss-Morris expanded on this finding, and suggested that while gastroenteritis can act as a trigger, psychological factors may be responsible for the maintenance of gut dysfunction over time [10]. In their prospective study of patients suffering from *Campylobacter* gastroenteritis, with no prior history of IBS, perceived stress and anxiety were identified as risk factors for symptoms of PI-IBS at 6 months post-infection [10]. In addition, severity of the initial infection, as measured by duration of diarrhea, presence of blood in the stool, abdominal cramps, and weight loss of at least ten pounds, resulted in increased risk [11]. The effects are persistent, as studies show a prevalence rate of 15.4% in a large cohort of PI-IBS patients at 8 years post-infection vs. 5.4% in matched controls within the same community [12]. The mechanisms behind the chronic gut dysfunction in this subtype of IBS patients have been extrapolated from a combination of clinical and animal studies; major contributors are believed to be altered neuromuscular function [13, 14], permeability [15], inflammation [16], and possibly changes in serotonin-releasing enteroendocrine cells [17].

Traumatic life events and ongoing stress has been suggested as an important factor in both the initiation and perpetuation of IBS. Significantly more female IBS patients reported childhood abuse compared to controls [18]. However, further studies by the same group showed that only psychological, and not GI symptoms, were higher in a childhood abuse-IBS group vs. non abuse-IBS patients [19]. This suggests that early life stress may indirectly contribute to IBS persistence by predisposing to higher levels of psychological

distress in adulthood. Other studies have shown higher levels of anxiety disorders in IBS patients with a history of abuse vs. IBS without a history of abuse [20]. Another interesting study group is patients exposed to severe traumatic life events during war. Veterans of the Persian Gulf War show higher levels of abdominal pain, diarrhea, and visceral sensitivity with co-morbid anxiety compared to civilian controls [21]. Furthermore, on-going life stress is an important factor in maintenance of disease in IBS patients, as chronic life stress (CLS) in adulthood has been shown to be a strong predictor of symptom intensity, and patients that experience CLS often fail to improve [22]. Thus, stress can be considered a risk factor for the instigation and continuation of IBS; however, the exact mechanisms remain unclear.

B. 1. ii. Psychiatric co-morbidities

In light of the prominent role of stress in IBS, it is not surprising that there is a high level of stress-related psychiatric disorders that show co-morbid expression with the condition. A study by Whitehead *et al.* indicated that 94% of patients with IBS have concomitant psychiatric disorders, especially major depression, anxiety, and other somatoform disorders [23]. Other studies that focus on anxiety and depression (AD) specifically show 38.6% and 38.6% prevalence for these conditions respectively (vs. 24.2% and 16.5% in healthy subjects) [24]. In this group of patients, depression was significantly associated with impairment in IBS-specific quality of life (QOL). A study from the Netherlands showed 30% and 33% prevalence for anxiety and depression, which were associated with higher symptoms score and lower QOL compared to unaffected IBS patients [25]. AD was observed in over 50% of self-reported female IBS patients in a study

that failed to find increased psychiatric co-morbidity in individuals with diabetes (i.e. a comparable chronic health disorder) [26]. Interestingly, one study showed that patients with recurrent major depression had significantly higher scores on the Gastrointestinal Symptom Rating Scale-IBS; however, the scores were comparable to controls when the patients were in remission [27]. One prospective study (n=2456) showed anxiety as a predictor of IBS development in the 15 month follow-up period [28]; however both of the conditions were diagnosed by questionnaire.

The main question that stems from these observations is: which condition came first? High levels of childhood trauma, and animal models such as the maternal separation (MS) model of brain-gut dysfunction, would suggest that exposure to stressor in early life alters stress-susceptibility and pre-disposes to gut dysfunction later in life [29]. However, not all IBS patients experience childhood trauma. Conversely, it is also possible that unpleasant IBS symptoms, and additional features of IBS, contribute to the expression of AD. Furthermore, the two scenarios are not mutual exclusive; a common pathophysiological mechanism may contribute to both conditions. It is likely that both situations occur in different groups of patients, and that bi-directional influences exaggerate the co-morbid expression of chronic psychiatric and gut disorders. Large scale prospective studies are needed to clarify this issue.

B. 1. iii. Stress and PI-IBS

The discovery of adrenergic signaling in *E. coli* offers an attractive theory to link host stress and the specific subset of IBS patients that can trace the onset of symptoms to an episode of infectious colitis. This subset, namely PI-IBS, has become more apparent

due to the growing threat to our food and water supply from bacterial pathogens in our environment. As previously mentioned, the presence of anxiety and depression, as well as severity of the initial infection, independently increases the likelihood of developing PI-gut dysfunction [9]. Intriguingly, it is possible that these two independent risk factors share a common etiology.

Recent studies have shown that enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, has the ability to upregulate the expression of certain genes in response to the human stress hormone norepinephrine (NE) [30]. This phenomenon has also been witnessed in additional of bacteria at both the genus and species level including *Shigella*, *Salmonella*, *Pseudomonas auruginosa*, *Campylobacter jejuni*, as well as others [31-33]. The specific EHEC genes that are affected by the *in vitro* administration of NE are encoded within the locus of enterocyte effacement (LEE), and are associated with the pathogenøs ability to cause attaching and effacing (AE) lesions on the host epithelium [30].

Hence, bacteria may utilize signals released by the host as a means of gauging their environmental surroundings, allowing them to determine if the situation is conducive to their growth and survival. Signals released during stress may indicate a period of host susceptibility, allowing enteric bacteria to coincide the metabolically costly expression of virulence genes with host susceptibility. However, this relationship has not yet been seen *in vivo* (i.e. an animal model of stress and infection), and adrenergic signaling has not been shown in non-pathogenic bacteria.

B. 1. iv. Motility and barrier function in IBS

Intestinal dysmotility is commonly reported in IBS patients, and is often reflective of their respective subtype (i.e. IBS-C or IBS-D); however, the role of motor disturbances in the pathophsyiology of IBS in unclear [34]. Ambiguity in this area of research is compounded by methodological difficulties, a heterogeneous patient population, and an imperfect correlation between motility measures and symptom expression [35]. Nonetheless, studies have indicated alterations in distal colonic motor activity [36], colonic propulsive activity [37], altered small intestinal motility (43% of patients) [38], and delayed gastric emptying (39% of patients) with concomitant small intestinal motor dysfunction [39]. In a recent study by Camilleri *et al.*, abnormal colonic motility was cited as the most prevalent physiologic abnormality in a group of 122 female patients [40]; however, only 32% of the patients had a significant alteration. Additionally, gastric and small intestinal transit was unaffected in this study. A unique study by Latimer *et al.* showed significant difference between IBS patients and a normal control group in the number and duration of colonic contractions; however, the difference was lost when the IBS group was compared to a separate non-IBS control group that was matched for psychological disturbances[41]. Therefore, it seems likely that intestinal dysmotility does occur in most IBS patients at some point in the course of their disorder, and it may contribute to symptoms of altered bowel habit. However, it may not be present in all patients, and additional influences may play a significant role in the observance of this phenomenon.

Intestinal permeability has also been examined in IBS patients. The intestinal barrier forms the interface between the host and the plethora of bacterial, viral, and

dietary antigens that reside in the GI tract. Equally, it is also responsible for the absorption of nutrients and the controlled sampling of potential pathogens in the gut. Regulation of the barrier is tightly controlled and disruptions can contribute to the expression of disease. In general, permeability is increased in IBS patients, but some discrepancies have been shown. Furthermore, subtypes may not be equally affected. One study showed increased proximal small intestinal permeability in atopic IBS patients, IBS-D, and PI-IBS [42]. Another demonstrated a significant mean increase in IBS-D, but less than half of the patients were above the threshold set by the controls (lactulose / mannitol ratio ×0.07) [43]. Marshall *et al.* noted a subtle increase in permeability in patients with sporadic and self-reported PI-IBS (lactulose / mannitol ratio ×0.02) [15]. Increases have also been seen using *in vitro* measures. Biopsies from IBS patients showed increased permeability with a concomitant decrease in tight junction proteins that correlated to symptoms of abdominal pain[44]. Similar experiments revealed increased rectal permeability in IBS-D [45]. In contrast, *in vivo* intestinal permeability was normal in 29 of 33 IBS patients (88%) in a heterogeneous population (lactulose / mannitol ratio < 0.025) [46]. Finally, simultaneous increases in permeability and inflammatory status were seen in PI-IBS patients up to 12 weeks post-infection [16]. Therefore, it appears that altered permeability is consistently observed in IBS-D and PI-IBS specifically; the latter suggesting that inflammation may play a role in barrier function in some patients.

B. 1. v. Dysbiosis in IBS patients

Only recently have we begun to understand the true importance of gastrointestinal microbiota to host health and well being. There are more bacteria in the GI tract than

somatic cells in the body and the two vastly contrasting environments are separated by a single layer of epithelial cells. Accordingly, many immunological and physiological mechanisms have evolved in an attempt to ensure an amicable relationship between hosts and their bacterial congregations. In health, it is a mutually beneficial relationship, with the microbiota capable of metabolic activities equal to a õvirtual organö (e.g. vitamin synthesis, fermentation of non-digestible food stuffs), providing essential nutrients to the host [47]. In turn, the bacteria are supplied with a nutrient rich environment in which they can thrive. Additionally, commensal (resident) bacteria in the GI tract have been shown to control intestinal epithelial cell differentiation and proliferation, metabolize dietary carcinogens, and play a role in epithelial barrier fortification [47]. Microbiota also play an integral function in host defense via pathogen displacement, production of antimicrobial factors, induction of protective immunoglobulins, and maturation and maintenance of the mucosal immune system [47]. Accordingly, alternations in the balance of host microbiota, or dysbiosis, have far reaching effects on host health and the prevention of disease both in the GI tract and beyond.

A role for microbiota in the pathogenesis of IBS stems from three main observations: 1) some patients develop IBS following acute bacterial gastroenteritis (PI-IBS, as previously discussed); 2) therapies that influence bacteria have shown efficacy in IBS, including prebiotics, probiotics and antibiotics; and 3) IBS patients have altered microbiota compared to healthy controls (HC) [48]. The latter observation has garnered much attention as of late, but significant work is still required before this issue is truly understood.

Early observations in this area were based on abnormal hydrogen production in breath-tests in IBS patients [49], however, most recent work has been completed using molecular methods. In 2005, Matto *et al*. showed temporal instability in IBS patients using denaturing gradient gel electrophoresis (DGGE) [50]; however patients using antibiotics were not excluded. This group later demonstrated decreased levels of *Clostridium coccoides-E. rectale* in IBS-C [51]. Additional studies have shown decreased diversity at a single time point using DGGE [52]. A study that used real-time PCR to asses over 300 species showed fewer *C. coccoides* subgroup and *Bifidobacterium catenulatum* group in IBS; subtype analysis showed lower *Lactobacillus* spp. in IBS-D, and increased *Veillonella* spp. in IBS-C [53]. Further study of the IBS-D subtype using clone libraries showed an increase in Proteobacteria and Firmicutes, with concomitant reduction in the number of Actinobacteria and Bacteroidetes [54]. A recent study by this group showed a *Ruminococcus torques*-like phylotype (a mucin degrading *Clostridium*) that associated with severity of bowel symptoms in IBS [55]. In addition to the predominantly studied fecal communities, Carroll *et al*. included mucosal samples in their 2011 study. In agreement with others, they showed lower fecal biodiversity in IBS-D; however, they did not find a difference between mucosal communities in IBS-D vs. HC [56]. Some recent studies that have focused on opportunistic pathogens have shown increases in *Pseudomonas aeruginosa* in the small intestine and feces of IBS [57], and the presence of *Staphylococcus aureus* [166]. Of note, most of the studies mentioned observed significant variation between individuals, even in the health.

The aforementioned studies are by no means exhaustive for this field of research, and while differences between IBS and HC are repeatedly shown, the exact consequences of these changes, and the specific bacteria involved, remain uncertain. Bacteria from the phylum Firmicutes seem to contribute to many of the differences, with changes to the families *Lachnospiraceae* and *Ruminococcaceae* and the genera *Streptococcus, Lactobacillus*, and *Veillonella* repeatedly observed [48]. Continued observation of these changes will strengthen the concept that they may play a role in GI health and disease.

B. 2. The relationship between motility and microbiota

Although it is intuitive to think that changes in intestinal motility will change the intestinal microbiota, most of the evidence is indirect. For example, numerous studies have shown changes in breath testing in patients with gastrointestinal dysmotility [58-61]. Also, small intestinal bacterial overgrowth (SIBO), albeit controversially, is often associated with IBS [62, 63]. One study showed changes in hydrogen and methane production in patients with gastroparesis (60%), indicative of bacterial overgrowth. Furthermore, patients with a longer duration of dysmotility were more likely to have SIBO [59]. A study by Vantrappen *et al*. examining the interdigestive motor complex (IMC) in man [a.k.a migrating motor complex(MMC)] revealed that all of the subjects with disturbed or absent motor complexes had abnormal breath tests [64]. However, determining cause and effect in these situations is challenging. Directionality is suggested by animal experiments in which pharmacological disruption of the IMC in rats resulted in higher levels of bacteria in the small bowel [65]. Interestingly, the overgrowth was not longer present when the drug became inactive and the IMC returned to normal.

Evidence for a reciprocal influence also exists (i.e. changes in bacteria may alter motility). Early work in germ free mice show alterations in enteric nervous system structure that could theoretically influence motility [66]. Later germ free work in rats showed that the introduction of conventional microflora could alter myoelectric activity of small intestine [178]. This group expanded on these findings using monocolonization methods, showing that this effect was species specific, and that come species increased the MMC period (eg. *Clostridium tabificum*), while others decreased it (eg. *Lactobacillus acidophilus* and *Bifidobacterium bifidum*)[177]. A variety of pathogens have demonstrated the ability to modulate intestinal motor parameters, including *Vibrio cholerae,* various strains of *Salmonella typhimurium*, and toxigenic *E. coli* [64]; however, these effects could be specific to disease-inducing pathogens and not applicable to commensal organisms. It is likely that inflammatory sequel from the infection, and not the bacteria themselves, are responsible for the aberrant motility [67, 68].

Regrettably, many of these observations are made in the context of disease and confounding factors are likely. Also, data from studies that utilize molecular methods is lacking. Clinical experiments with a prospective design will help clarify this issue in humans.

B. 3. The stress response

In 1955 Hans Seyle, a pioneer in stress research, reminded us that is it not the exposure to noxious stimuli itself that results in the expression of disease, but rather our reaction to it [69]. In this context, stress can be defined as any stimuli that threatens the homeostasis of an organism, whether it be real (physical) or perceived (psychological).

The stress response is the organism's attempt to regain the stability of the system, and is intended to limit damage and promote survival. However, chronic activation of the stress response can become maladaptive and lead to the expression of disease.

Centrally, exposure to stressors results in prefrontal cortex-mediated activation of both the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the sympathetic branch of the ANS originates in the locus ceruleus/norepinephrine nuclei within the brainstem (LC/NE system). In the central nervous system (CNS), locally released NE results in heightened arousal and vigilance. Communication with the periphery occurs via efferent fibers that leave the CNS and terminate in ganglia adjacent to the spinal column. Post-ganglionic fibers extend to various tissues and release norepinephrine (NE), or NE and epinephrine in the case of the adrenal glands. Generally, exposure to stressors decreases output from the parasympathetic branch of the ANS; however, some parasympathetic nuclei show heightened activation [70].

Activation of the HPA axis begins with the production and release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus, as well as other brain regions. CRH stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which in turn stimulates the release of glucocorticoids from the adrenal glands (primarily cortisol in humans, corticosterone in rodents) [71]. Further activation occurs locally, with serotonergic and noradrenergic neurons, from the raphe nuclei and LC respectively, providing positive feedback activation of the ANS and HPA axis [72]. Accumulating evidence has shown that

the HPA axis can also be activated by inflammatory cytokines such as IL-6, IL-1 and TNF-α [73].

Termination of the stress response is initiated by signals from both the CNS and the periphery. Centrally produced compounds, such as CRH and ACTH, provide short feedback inhibition. Additionally, glucocorticoids from the periphery, which bind receptors in the prefrontal cortex and hippocampus, exert an inhibitory influence [74]. Chronic exposure to stress can result in maladaptive alterations including increased production of CRH, altered sensitivity to NE, and downregulation of glucocorticoid receptors involved in negative feedback control [74].

B. 4. The effects of stress on the GI tract in humans

Stress impacts the GI tract in both health and disease. High levels of stress has been associated with the onset and exacerbation of many GI disorders including gastroesophageal reflux disease, functional dyspepsia, peptic ulcer, inflammatory bowel disease and IBS [74]. The direct influence of stress on these conditions is often unclear, and is likely due to a complex interaction between the many components influenced by stress, including GI physiology, endocrinology and the immune response. To understand these multifaceted disease conditions we must first identify the effects of stress on the GI tract in health.

Two of the most well studied aspects of GI physiology that are influenced by stress are motility and barrier function. There are a limited number of studies looking at the effect of psychological stress on GI motor function in healthy human volunteers and

the numbers of subjects are often quite low. Nevertheless, Rao *et al.* showed that both physical (cold) and psychological (dichotomous listening) stress affects colonic pressure activity, with the psychological stressor resulting in a more prolonged response [75]. Others have shown stress and GI associations including: cold stress and inhibition of gastric emptying of liquid meal [76]; shock avoidance and dysrhythmic gastric myoelectrical activity [77]; 30 minute stress session and altered normal postprandial gastric myoelectrical activity [78]; as well as prolonged active coping stressor and decreased small intestinal transit [79]. Even fewer studies have been completed looking at changes to barrier function; however altered lactulose/mannitol permeability has been noted in patients under extreme conditions including surgical stress[80], and burn trauma [81]. Together, these studies show that GI physiology can be modified by a wide range of stressors in humans and that the effects vary in different areas of the GI tract.

B. 5. The effects of stress and CRH on the GI tract: animal models

The vast majority of work looking at stress and GI function has been completed in animal models. Experimental exposure to stressors in rodents recapitulates most of the physiological changes highlighted above. Additionally, the effects are mimicked by administration of CRH and can be block with a CRH-antagonist.

B. 5. i. Motility

Much like humans, stress has been consistently shown to alter GI motility in a region specific manner in animals. In general, exposure to both physical and psychological stressors leads to decreases in both gastric and small intestinal motility,

with concomitant increases in colonic motor function. Early work by Taché and others showed that microinfusion of CRH into the PVN of the hypothalamus was able to reproduce the delayed gastric emptying and increased colonic transit evoked by restraint stress in a dose-dependent manner [82]. Furthermore, the effects of restraint stress were blocked by the antagonist α-helical CRF-(9-14). Vagotomy incompletely blocked the effects of CRH in both the stomach and the colon; however, atropine (parasympatholytic) was able to completely abolish the effects on the colon. This affect does not appear to involve down-stream mediators of the HPA axis since colonic motor dysfunction induced by stress or CRH is not prevented by hypophysectomy [83]. Additional stressors, including swim stress, abdominal or cranial surgery, visceral irritation, ether stress, and injection of IL-1β, have induced GI motor changes that have been blocked by a variety of CRH antagonist[84].

Further characterization of the mechanisms involved in CRH-accelerated colonic transit revealed that it is mediated by increased parasympathetic outflow since it can be blocked by peripheral injection of ACh receptor antagonists (atropine and chlorisondamine), but not a sympatholytic (bretylium) [85]. This was later verified in rats exposed to water avoidance stress, which showed significantly higher Fos expression in the PVN and the parasympathetic nucleus of the lumbrosacral spinal cord [70]. Increases in colonic serotonin, and binding of serotonin receptors, has emerged as the final result of the increased parasympathetic outflow induced by central injection of CRH [86, 87].

Interestingly, not all areas of the GI tract are controlled in the same manner. A study by Nakade *et al.* used restraint stress to show a CRH-mediated delay in gastric

emptying. This effect was blocked by drugs that reduced the release of NE (guanethidine) and block NE binding (propranolol; -adrenergic antagonist), as well as celiac ganglionectomy [88].

Finally, peripheral injections of CRH are also able to replicate stress-induced changes in motility, which can be blocked by the CRH antagonist astressin [89, 90]. However, the relative contributions of central vs. peripheral CRH is unclear, and the exact mechanisms behind the effect of peripherally injected CRH are controversial [91]. Some evidence suggests that stress (including exposure to bacterial toxins) can increase the expression of CRH receptors in the gut [92]. It is possible that peripheral CRH may play a more prominent role in controlling gut motility under certain pathological conditions.

Together, this work shows that CRH is responsible for stress induced changes in GI motility, and that the final outcome and mechanism involved varies depending on the region of the gut.

B. 5. ii. Barrier function

Fewer clinical studies have been completed looking at the effects of stress on the intestinal epithelial barrier; however animal models have consistent shown increases in permeability in response to experimental stressors. Specifically, restraint stress in rats showed increased permeability to [^{3}H] mannitol and [^{51}Cr]-labeled EDTA[93] *ex vivo*, indicating increased transcellular and paracellular permeability respectively. Later work by Santos and others demonstrated that water avoidance stress resulted in increased permeability to the macromolecule HRP; an effect that was not seen in mast cell deficient mice [94]. Moreover, peripheral administration of CRH duplicated these changes, and a

CRH-antagonist inhibited them [95]. The mechanisms behind these affects appear to be dependent on mast cells, which may be activated by ACh and/or CRH from the periphery [96]. Importantly, in contrast to the stress-induced changes in motility, stress-induced alteration in permeability are not region specific (i.e. it increases in all sections of the GI tract), and can be dependent on the integrity of the HPA axis[97].

B. 6. Stress and immune modulation

There is extensive bi-directional communication between the ANS, HPA axis and the various components of the immune system that is facilitated by a shared network of signaling molecules and receptors. Overall, the nature of the immune response will be influenced by the character and the duration of the stressor. Ultimately, the goal is to promote the survival and health of the host, however, chronic exposure to stress can increase susceptibility to some types of disease.

The most well recognized cross-over between the two systems is orchestrated by the end product of HPA axis activation. Glucocorticoids (GC) have long been recognized for their anti-inflammatory and immunosuppressive properties; effects that are initiated at the molecular level through the modulation of a multitude of glucocorticoid-responsive genes [98]. GC modulate the immune response via a variety of effects including: suppression of cytokine production, release, and efficacy (particularly T_{H1} cytokines), altered lymphocyte trafficking and proliferation, and induction of tolerogenic dendritic cells [99]. However, as previously mentioned, chronic activation of the HPA axis can result in functional changes to the system. For example, veterans with post-traumatic stress disorder, a condition associated with HPA axis hyperactivity, show decreased leukocyte

GC receptor (GR) density. Additionally, when exposed to a synthetic GC, these patients had reduced inhibition of T cell proliferation, and decreased production of the anti-inflammatory cytokine IL-10 [100]. Furthermore, individuals with clinical depression, another condition with HPA axis dysregulation, also show reduced GR sensitivity and deficits in GC-mediated immune suppression [101].

While the HPA axis is the most widely studied, other components of the stress response system exert immunomodulatory effects. Lymphoid organs in the periphery have extensive sympathetic innervations, and NE and epinephrine released at these sites are able to bind adrenoreceptors on various immune cells. In general, the influence of the SNS is immunosuppressive and shows much overlap with the HPA axis. For example, much like GC, catecholamines suppress cytokine production. Specifically, they can inhibit IL-12 production by antigen producing cells and subsequent production of T_{H1} cytokines such as IFN-γ, IL-1, and TNF-α [102]. However, evidence suggests that under certain conditions catecholamines may stimulate local immune responses, allowing for more tailored regional control. [103].

Finally, stress-induced CRH has also been shown to have immunomodulatory effects in the periphery. Mice exposed to chronic restraint stress demonstrated CRH expression in intestinal eosinophils [104] and splenocytes [105]. Many types of immune cells bare receptors for CRH, and binding generally results in a proinflammatory response. Specific effects include increased cytokine production by macrophages [106], and mast-cell degranulation [107].

Together, these findings demonstrate extensive communication between the ANS, HPA axis and the various components of the immune system. In general, activation of the stress response results in an adaptive downregulation of inflammation, however, local stimulation may occur under some circumstances. Additionally, excessive or prolonged activation may result in a maladaptive immune response and susceptibility to disease.

B. 7. Stress and the microbiota: Animal models of host stress and GI dysbiosis

The introduction of molecular techniques to study bacterial communities in animal models has bolstered the longstanding observation that stress can change bacterial communities in the GI tract. Classic culture techniques used in a variety of animal models including environmental and dietary stressors [108], restraint stress [109], and both pre- and post-natal stressor showed significant alterations in GI microbiota at the genus level [110, 111]. More recently, using both culture techniques and 16s rRNA based pyrosequencing, Bailey *et al*., showed that prolonged restraint stress (7 days, 14 hours/day) resulted in an overall decreased richness and diversity, and a specific reduction in a member of the Bacteroidetes phylum (family *porphyromonadacae*) [112]. This study included food and water deprivation controls (FWD) to strengthen the argument that the change was due to the stressor, and not environmental variations. Of note, when home cage controls were compared to FWD controls there were differences in the Firmicutes phylum (families l*achnospiraceae* and *ruminococcaceae*), indicating that these variables have a significant effect on the microbiota. Furthermore, the stress-induced changes resulted in an increased susceptibility to infection with the pathogen *C. rodentium*. Another study by this group

using social disruption stress (6 days, 2 hours/day) demonstrated reduced diversity and richness, and confirmed stress-induced decreases in the Bacteroidetes phylum (family *bacteroidaceae*; species *bacteroides*) [113]. There was also a nonsignificant increase in Firmicutes (genus *clostridium*) (p=0.07). Molecular techniques have also verified earlier studies that suggested that post-natal stress can shape the microbiota. O'Mahoney *et al.* used DGGE to reveal significant changes to the fecal bacteria in the maternal separation model of early life stress[29].

While some of the animal models noted above demonstrate consistent changes at the phylum level, and begin to clarify the temporal relationship between stress and dysbiosis, the mechanisms that result in stress-induced changes to microbiota are still unknown. Determining how these changes occur is vital to the development of strategies to combat stress-induced dysbiosis and maximize host health.

B. 8. The olfactory bulbectomy (OBx) model

B. 8. i. Behaviour

Many of the behavioural changes that occur following OBx can be described as behavioural disinhibition [114]. The classic behavioural assessment of OBx is hyperactivity in a novel open field, but other tests have been used that show similar changes in activity, exploration levels and reactivity to stress. Other reported behavioural alterations include: hyperactivity in a novel home cage [115]; increased exploratory behaviour in a novel object test and T-maze in mice [115]; reduced time spent in the center of an open field in mice [115]; increased rearing, self-grooming, and defecation in an open field in rats and mice [116-119]; increased exploratory head-dipping behaviour in the hole-board test in mice [120];

hyperactivity in forced swimming test in mice [121], impaired spatial and short term memory [122]; increased predatory aggression paired with decreased intraspecies aggression in mice and rats [121-123]; and deficits in passive avoidance in mice and rats [119, 123, 124]. Other behavioural changes that have been noted and subsequently compared to depressed patients include: reduced libido in rats (quantified by analyzing genital probing and thrusting behaviour)[117], decreased social interaction in rats [117], and altered circadian rhythms of motor activity and body temperature in rats [125]. Some measures such as the elevated plus maze has shown conflicting results. Song and Leonard, 1994, showed increased time in the open arms and decreased time in the closed arms, and attributed the results to an increase in exploratory behaviour [118]; conversely Wang *et al.*, 2007, showed a decreased ratio of time exploring the open arms to the total test time and interpreted the results as an increase in anxiety levels in the OBx mice [117]. These studies highlight the importance of using a battery of tests to characterize behavioural changes, and warn against the over extrapolation of single measures of animal behaviour to complex human constructs such as anxiety and depression. Additionally, the amount of time spend handling the animals following surgery can greatly affect the degree of behavioural change and must therefore be kept constant between trials; this factor could also account for differences in behavioural tests between investigators [126].

A common factor between many of the behavioural responses of the OBx model is that they are most apparent when the test are conducted under conditions that are associated with increased stress. For example, rats tested in an open field that has low

lighting and transparent walls will not exhibit the same hyperactivity as those tested using an apparatus that has reflective walls and bright lighting [126].

Thus, the combination of hyperactivity and hyperemotionality described above has lead to the specialization of OBx as a model of agitated depression with comorbid anxiety-like behaviour.

B. 8. ii. Endocrine system

A change in the level of the stress hormone corticosterone (CORT) is perhaps the most controversial measure in the OBx model. Some studies report no significant change [121], while others report an increase in serum CORT levels [125]. One group reported decreased levels of CORT in bulbectomized female rats, however no male controls where included in the study [127]. Some of the differences in findings appear to be related to the method of testing; more specifically the time of day that the blood is taken. This issue was addressed in a study by Song *et al.*, 1994, which looked at serum levels of CORT every four hours over a twenty-four hour period [128]. This more detailed analysis showed that while serum CORT levels in OBx rats followed the same circadian rhythm as controls (nocturnal increases), there was a significant hypersecretion of CORT during the dark phase as compared to controls; other time course experiments have found similar results [125]. Accordingly, the studies that took serum measurements during the day may have missed the difference in OBx animals.

While disruptions in basal levels of ACTH and CORT following OBx are unclear, it seems that levels of this hormone are higher in bulbectomized rats under conditions of stress. Ether stress has been shown to increase both CORT and ACTH in OBx animals as

compared to sham controls [129]. Furthermore, this report also showed that basal POMC (pro-opiomelanocortin; a precursor to ACTH) expression was increased more than 60% in OBx animals. An increase in basal POMC expression paired with high basal CORT levels suggest that the feedback control of HPA may be altered in the OBx animals [129]. Further studies on possible changes to the HPA axis in OBx animals are limited, although one study failed to find an increase in the area or intensity of CRH-immunostaining or CRH mRNA levels in the external layer of median eminence [130].

B. 8. iii. Neuroanatomy

The removal of the olfactory bulbs results in a cascade of changes within the CNS that are far reaching. Removal of the OBs results in retrograde degeneration of the neurons that have inputs into the OBs, as well as anterograde degeneration of neurons that leave the OBs and project into other areas of the brain [114]. The system within the CNS that seems to be the most affected by its altered input from the OBs is the limbic system. Consequently, it has been credited with many of the behavioural changes seen in the OBx model [131]. The limbic system controls emotional behaviour, motivational drives and long term memory, and includes structures such as the hypothalamus, thalamus, hippocampus, and amygdala [132]; the latter of which is believed to have the greatest influence on behaviour in the OBx model [131].

The overall function of the amygdala is to integrate environmental input and learned experiences in an attempt to pattern behaviour in a way that is deemed suitable to the present situation [132]. Many of the behavioural alterations seen in the OBx model can be interpreted in a meaningful way with this function in mind. A magnetic resonance

imaging (MRI) study of OBx rats by Wrynn *et al.* in 2000, showed a significant decrease in the signal intensity from the amygdala, indicating a decrease in volume [133]. Subsequently it has been suggested that the OBs provide inhibitory input into the structure and that loss of this connection results in a failure to inhibit anxiety-like behaviour in response to a novel environment [114]. Comparatively, neuroimaging studies have been done in human patients during major depressive episodes, which are described by Drevets, 2003, as õpersistent dysphoric, anxious, and irritable emotional experiences and thoughts that coexist with disturbances of motivation, social behaviour, sleep, and psychomotor activityö; these studies indicate alterations in resting cerebral blood flow and glucose metabolism in the amygdala [134]. Subsequent studies in mice have shown neurodegeneration in the amygdala via increases in Fluro-Jade B staining; an effect that was reversed by treatment with amitriptyline [119].

MRI studies of OBx rats have also shown a decreased signal intensity from the cortex (frontal, occipital, and cingulated), and the caudate [133]. These studies also report an increase in the volume of the lateral and third ventricles; a feature that has been noted in depressed human patients as well [133]. Moreover, the images showed alterations in the area of the hippocampus; unfortunately, the exact nature of the changes was unclear due to the hyperintensity of adjacent lateral ventricles. These findings are pertinent in relation to the large body of evidence supporting changes in the hippocampus in major depression [135-138]. Interestingly, this study also showed increased deposition of magnetic nanoparticles at the PVN of the hypothalamus, suggesting a focal disruption of the blood brain barrier at this point. Lastly, degeneration of the neurons projecting to the locus coeruleus and the dorsal

raphe nuclei also occurs; A feature which may account for the altered noradrenergic and serotonergic systems respectively (see below).

B. 8. iv. Neurotransmitters

The majority of treatments for major depression in humans are aimed at rectifying imbalances in monoamines in the CNS; specifically serotonin and NE [139]. Accordingly, these compounds have received the majority of attention in OBx studies looking at neurochemical changes in the brain.

Some researchers have expanded the categorization of the OBx model to that of a model of agitated hyposerotonergic depression [140]. This idea receives supports from the findings that many changes following bulbectomy can be reversed by selective serotonin reuptake inhibitors such as fluvoxamine [118]. Studies by Lumia *et al.* reported a parallel increase in 5-Hydroxyindoleacetic acid (main metabolite of serotonin) and decrease in 5-hydroxytryptamine (5-HT; aka serotonin) suggesting that there is a reduction in serotonin turnover in the frontal cortex, nucleus accumbens, hippocampus and corpus striatum [140]. Additional studies have shown decreased serotonin pools in the amygdala and the hippocampus; the latter being evident as late as 5 months post-surgery [141]. Marcilhac *et al.* also showed decreased basal levels of 5-HT in the amygdala. In contrast, the same study recorded stress-induced increases in serotonin in the hypothalamus with concomitant HPA axis hyperactivity vs. stressed sham controls [142]. These findings suggest increased and decreased activity in the hypothalamus and amygdala respectively, and underline the importance of studying components of the CNS in a region specific manner.

Several changes are also seen in the noradrenergic system including: reduced NE levels in the amygdala [118], increased affinity of the β-adrenoceptor in the amygdala and hippocampus, but not the cerebral cortex [143], and increased density of β-adrenoceptors on blood lymphocytes and the amygdaloid cortex [144].

In conclusion, many neuroanatomical and neurochemical changes have been shown in the CNS following OBx; many of which are seen in depressed patients as well. However, the overall functionality of these systems is often ambiguous as many of the studies fail to assess all of the components in a particular animal at a particular time point; i.e. looking at the level of the neurotransmitter and its metabolites, as well as the quantity and binding capacity of its associated receptor.

B. 8. v. Immune response

Immunological understanding of the OBx model is in its infancy; however Leonard and Song, leaders in the field, have hypothesized that OBx animals may have an immune status that is generally pro-inflammatory, with increased sensitivity to and/or increased production of IL-1β, IL-6, and TNF-α, and depressed IL-10 [114, 131]. They have also reported reduced thymus and spleen weights; a decrease in neutrophil phagocytosis; an increase in monocyte/macrophage activity; and increased PGE2 production [114, 131]. Furthermore, these findings are consistent with depressed patients [114, 145]. Perhaps one of the most relevant finding in OBx mice in regards to enteric infections is the reported response to lipopolysaccharide (LPS); while basal levels of pro-inflammatory cytokines are elevated, OBx rats have a blunted IL-1β and TNF-α response to the component of

gram-negative bacteria cell walls [146]. Additionally, treatment with the antidepressant desipramine was able to normalize behavioral perturbations in this study, but further impair the immune response to LPS. This calls to attention the fact that some treatments may only target certain alterations in OBx animals, and that more than one biological mechanism may be responsible for the changes that occur following bulbectomy.

Other immunological findings include concomitant increases and decreases in the percentage of neutrophils and lymphocytes respectively [118, 121, 128], and suppressed lymphocyte proliferation in response to mitogen (PHA and ConA) [118]. It has not yet been shown whether anosmia can induce the same changes [145].

Few studies has been completed that look at disease susceptibility in the OBx rat. In 2006, Breivik *et al.* showed a significantly worse disease course in an animal model of periodontitis, which was reversed by tianeptine [116]. In concurrence with Conner *et al.* [146], this study also showed blunted TNF-α responses to LPS following OBx. Specifically in the GI tract, one study used the OBx model to has show that quiescent colitis can be reactivated following the induction of depression [147], suggesting that OBx per say can influence GI immunity. However, the first intercession in this study was the colitis, not the depression, and the impact on the microbiota was not addressed.

B.9. Comorbid anxiety and depression

Early versions of the Diagnostic and Statistical Manual of Mental Disorders created by the American Psychiatric Association attempted to disconnect depression and anxiety as two different clinical entities that could not co-exist. Strong challenge from professionals in the field, supported by epidemiological studies, lead to the revision in

later versions of the manual that allowed for the concomitant diagnosis of anxiety and depressive disorders [148]. A recent study indicated that 20.1% of patients with a single anxiety disorder also suffered from major depression disorder (MDD); Moreover, patients with 3 or more anxiety disorders had a MDD comorbidity rate of 87% [149]. Further support for a significant overlap between the two conditions comes from the observation that many pharmacological antidepressant treatments are also effect anxiolytics[148]. Additional shared features include: varying degrees of HPA axis dysregulation (stronger association in depression), shared genetic factors, and exposure to traumatic life events, especially in childhood [150, 151]. Interestingly, in a study by Young *et al.* patients with co-morbid anxiety and depression showed significantly elevated HPA axis activity (serum ACTH) in response to a mild social stress test (vs. matched controls), while subjects with mood or anxiety disorders alone did not. The authors suggested that simultaneous activation of both the NE/LC system and the HPA axis may play a role in this specific group of patients [152].

Thesis Statement:

This thesis examines how aberrant communication within the gut-brain axis, in the form of host stress, can influence host health and susceptibility to disease

Aims and Hypothesis:

The specific aims of this thesis were to determine:

I. If psychological stress could increase NE levels in the gastrointestinal tract.

II. If ten days of psychological stress can influence the outcome of bacterial gastroenteritis.

III. If chronic psychological stress, in the absence of environmental modifications, can lead to changes in the GI microbiota of adult mice.

IV. Which mechanisms contribute to stress-induced changes in the microbiota.

The specific hypothesis of this thesis were:

I. Psychological stress will increase colonic NE levels and worsen the outcome of acute bacterial gastroenteritis; ultimately, this will exacerbate the degree of post-infectious gut dysfunction

II. Chronic activation of the stress response will alter the GI microenvironment and result in changes to the microbiota

III. Neural or immune-mediated mechanism will contribute to alterations in physiology and the microbial habitat; ultimately, these changes will contribute to stress-induced dysbiosis

Materials and Methods:

The methods used within this thesis, and the design of the experiments, were chosen to minimize variables that may directly influence the microbiota and thus confound the specific influence of host stress. In all experiments, attempts were made to minimize contamination and environmental modification. Specifically: the bacteria for the enteric infection was chosen because it is a natural murine pathogen and therefore does not require pre-treatment with antibiotics; we did not use stress protocols that involved significant interruptions to food and water availability, changes in bedding, or contact with novel animals; introduction to novel environments was minimized, and when necessary (i.e. behavioral testing) all equipment was cleaned between each animal; and finally, the model of psychological perturbation was chosen because it did not require constant behavioral/environmental stressors (e.g. restraint stress) or pharmacological treatments that might influence the microbiota (e.g. catecholamine depletion), and did not require any post-surgical manipulation of any kind for the maintenance of chronic depression co-morbid anxiety.

Animals: Male and female C57BL/6 mice were purchased from Taconic (Hudson, New York, USA) at 8-10 weeks old and housed in a specific pathogen- free unit at the McMaster University Central Animal Facility. Mice were allowed to acclimatize for a minimum of one week before any experiments were completed. All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and received approval from the McMaster University Animal Research Ethics Board. Male

mice were used for *C. rodentium* experiments. All bulbectomy experiment were conducted using female mice.

Bacterial Infections: Overnight cultures of wild type *Citrobacter rodentium* (DBS100; ATCC 51459) were grown in luria broth (LB; Sigma-Aldrich, Oakville, Ontario, Canada) at 37°C. The following morning the cultures were centrifuged and the bacterial pellet was washed in sterile LB. This process was repeated for a total of two washes, and then the pellet was resuspended in sterile LB to a concentration of 10^9 CFU/ml (optical density=1.0). 0.1 ml of this solution was administered to the mice via orogastric gavage using a blunted 0.22 gauge needle. Enumeration of the bacterial load was completed using standard plating techniques on brilliant green agar (EMD Chemicals, Gibbstown, New Jersey, USA). In stress experiments, mice were exposed to 10 stress sessions starting the day they were infected. Immune responses were assessed at two end-points: day 11 (approximate peak of bacterial load) and day 18 (approximate peak of inflammatory response). Extreme care was taken in handling infected animals. No bacteria were ever recovered from control mice.

Water avoidance stress (WAS): One session of WAS (60 minutes) was used to assess stress-induced norepinephrine levels in the colon of male mice. Ten consecutive sessions (60 minutes each) were used to determine the effect of stress on *C. rodentium* infection in male mice. 10 minutes of WAS was employed to asses fecal output, c-fos, and 5-HT in female OBx mice. The mice were taken directly from their home cage and placed on 2 cm high platforms surrounded by sterile water (1 cm deep). Mice were separated from each

other by Plexiglas barriers, and the apparatus was sterilized between testing sessions to minimize contamination. Control mice were never in contact with *C. rodentium* infected mice, or the equipment/water they used.

Immune response to infection: The immune response to infection was measured using tissue cytokines. Measurement of cytokine levels were done in whole tissue (colon and mesenteric lymph nodes), which was homogenized in Tris HCl buffer containing protease inhibitors (Sigma-Aldrich). The supernatant was analyzed using individual Quantikine colorimetric enzyme-linked immunosorbent assays (ELISAs) as per the manufacturer's instructions (R&D Systems, Minneapolis, Minnesota, USA).

Olfactory bulbectomy (OBx): All surgeries were completed at 9-11 weeks of age and all additional measurements were completed at 8-10 weeks post-surgery. Mice were grouped housed with both surgical groups represented in each cage (n=2-3 OBx and 2-3 Sham per cage; maximum n=5 per cage). Experiments were completed with n=5-12 mice per surgical group. Prior to surgery, mice were anesthetised with ketamine (150 mg kg-1, i.p.; Bimeda-MTC, Cambridge, Ontario, Canada) and xylazine (10mg kg-1, i.p.; Bayer Health Care, Toronto, Ontario, Canada). The skin coving the frontal bones of the skull was shaved and sterilized, and a small incision was made using a scalpel blade. Two burr holes were made in the skull overlying the olfactory bulbs using a microdrill (steel burr; Meisinger, Centennial, Colorado, USA). The olfactory bulbs were visually identified and removed by aspiration using a blunt glass pipette attached to a vacuum pump. Sham operated mice received the same treatment except the olfactory bulbs were left intact. The

wounds were packed with surgical sponge (gel foam; Pfizer, New York, New York, USA), closed by suture and treated with antibiotic (Baytril, topical; Bayer Health Care). Following recovery, the mice received subcutaneous saline and an analgesic (buprenorphine 0.05 mg/kg; Reckitt Benckiser, Mississaugua, Ontario, Canada). Mice were monitored daily for up to two weeks post-surgery. Great care was taken to ensure consistent handling and treatment in all of the groups examined. The success of the surgery was confirmed using multiple methods including: expression of the hallmark behavioral trait (i.e. hyperlocomotion) as compared their individual control (i.e. sham) animal; a food finding olfaction test that removed animals that located the buried food pellet; and visual and/or histological verification of the surgery post-mortem. Any individuals that showed sign of damage to other areas of the brain were excluded from analysis (less than 1% of mice).

Behaviour assessment: Mice were exposed to a battery of tests for anxiety- and despair-like behaviour including tail-suspension test (TST), latency to step-down (SD), and open field (OF) behaviour. TST: TST was completed as previously described [153]. Briefly, mice were suspending by their tail approximately 50 cm from the base of a retort stand using masking tape, which covered the most distal 1cm of their tail, a short metal bar, and surgical gut. The test lasted 6 minutes and duration of immobility was measured as an indicator of despair-like behaviour. SD: Mice are individually placed on the center of a wire-mesh covered platform (10 cm diameter, 4 cm high) that rested on a black plexi-glass surface. The time for all four paws to step-down on the novel surface is recorded to

a maximum of 5 minutes. OF: behaviour in a novel open field was measured using the OF test package from Med Associates (27.3 cm x 27.3 cm arena) (Med Associates, Inc., St. Albans, Vermont, USA). Parameters considered include: total-distance travelled, percent distance in center zone, and rearing counts. All experiments were conducted in a quiet, well lit room, with one handler throughout. Pairs of mice, consisting of one OBx and one control sham, were tested at the same time when feasible.

Isolation of the paraventricular nucleus (PVN) of the hypothalamus: Whole brains were carefully removed upon euthenization and immediately frozen in isopentane (Sigma-Aldrich) cooled to -80°C using dry ice. Brains were stored at -40°C until they were transferred to a microtome cryostat with a chamber temperature of -20°C (Microm 550; Thermoscientific, Nepean, Ontario, Canada). Brains were placed in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, California, USA) and positioned for coronal sections. The brains were trimmed at a thickness of 80µM until the suprachiasmatic nucleus. Next, slices were cut at 8µM and every tenth slice was placed on a slide and stained with toluidine blue to visually identify the start of the PVN. Once this point had been identified one 100µM section was removed and a square area containing the PVN was manually excised using a razor blade (see figure 6), and placed in a sterile microtube cooled to -20°C. An 8µM section was cut and stained to confirm location, and then a second 100µM section was taken as cut as before. A final 8µM slice was stained to ensure that the entire section of tissue collected was in fact PVN. Tissue was frozen at -40°C until processed.

RNA isolation and real-time polymerase chain reaction (PCR): RNA was isolated from the brain tissue using RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada) and treated with RNase-free DNase (Qiagen) to remove contaminating genomic DNA. Reverse transcription was performed using 1μg of RNA and M-MLV Reverse-Transcriptase (Invitrogen, Burlington, Ontario, Canada). PCR efficiency and optimal annealing temperature for each of the primer pairs was tested via standard curve and thermal gradient experiments respectively using a MyiQ2 Real-Time PCR Detection System (BioRad, Mississauga, ON, Canada). The PCR condition used were as follows: 95° C for 3 minutes, followed by 40 cycles of 95° C for 10 seconds, 58° C for 30 seconds, and 72° C for 30 seconds. Amplification was performed in triplicate 20 l reactions and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The product size was confirmed by gel-electrophoresis and specificity of the amplification was tested by performing melt-curve analysis for all reactions. CRH primer sequence: forward 5ø-CAA CAG GAA ACT GAT GGA GAT T-3ø; reverse 5ø-GGA GCT GCG ATA TGG TAC AG-3ø. GAPDH primer sequence: forward 5ø-CCA TGG AGA AGG CTG GGG; reverse 5ø-CAA AGT TGT CAT GGA TGA CC. Gene expression was analyzed using the 2- Ct method.

Microbiota: Stool samples were collected by restraining the mice and collecting the first fresh pellet passed into a sterile microtube, which was immediately frozen in liquid nitrogen. Samples were stored at -80 until processed. Bacterial DNA was extracted using a Phenol/chloroform/isoamyl (PCI) method, paired with a Clean and Concentrator kit

(Zymo Research Corp., Irvine, California, USA). PCR was performed using HDA-1 and HDA-2 universal primers for the V3 region of the 16s rRNA gene. SequenceS: HDA1-GC- CGC CCG GGG CGC GCC CCG GCG GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T. HAD2- GTA TTA CCG CGG CTG CTG GCA C. The PCR products (rDNA) were separated based on their G+C content and distribution using denaturing gradient gel electrophoresis (DGGE). This technique was employed because it is an economical method that allows for the profiling of complex communities. It does not require the use of primers designed for defined target groups (e.g. fluorescence in situ hybridization), and allows for the analysis of multiple samples at one time (vs. clone libraries). It is used with the caveat that there may be imperfect resolution of the bands, and that it is subject to the many technical issues related to PCR including, but not limited to, DNA extraction bias, primer/dimer formation, PCR contamination and inhibition, etc.. Gels (35%-55% denaturing gradient) were subjected to a constant voltage of 130 V for 4.5 h at 60°C using a Dcode universal mutation detection system (Bio-Rad Laboratories, Richmond, California, USA.). After electrophoresis, the gels were stained for 20 min in 1× TAE containing SYBR Green (Molecular Probes, Eugene, Oregon, USA), photographed under UV illumination, and analyzed using Bionumerics software (Applied Maths, Austin Texas, USA). The Pearson correlation coefficient was used as it accounts for both presence/absence of a band as well as the band intensity [155, 156]. Statistical analysis was completed by running a script in the Bionumerics software that compares the within- and between-group similarities with randomized tests (1000 iterations)[157]. The P value represents how frequently the random similarity is greater than

the experimental similarity; $p<0.05$ represents a significantly distinct group. The dendrograms were constructed using the Neighbour-joining clustering method. This method builds a tree based on the principle of minimum evolution by starting with the two most similar samples, and then creates a branching pattern by adding branches of the shortest possible length (smallest amount of change between samples) until all individuals are included. It does not assume a constant rate of evolution/change as Unweighted Pair Group Method with Arithmetic Mean (UPGMA) does, and is considered to be more realistic and consistent [158].

Fecal output: Fecal output has been used as an indicator of gastrointestinal motility [159]. Defecation was initiated by exposing the mice to a mild stressor (water avoidance stress; see above). The total number of fecal pellets expelled during a ten minute session was counted.

Contractility: Mice were taken directly from their home cage and euthanized by cervical dislocation. Sections of colon were removed and placed in Krebs buffer containing (in mM) 120.9 NaCl, 1.2 NaH_2PO_4, 15.5 $NaHCO_3$, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, and 11.1 glucose. As previously described [160], two pieces of 5-10 mm silastic tubing (0.94mm; Dow Corning, Midland, Michigan, USA) were placed in the lumen at either end of the tissues to allow for proper oxygenation. A closed end loop of suture was placed at one end and attached to a tissue hook in the organ bath containing Krebs buffer at 37^oC under 95% O_2-5% CO_2. The other end of the tissues were tied with a long suture and attached to a force displacement transducer, which was then calibrated to a tension of 1 gram. The

tissues were then stretched to optimal tension (800mg) and then allowed to equilibrate for 30 minutes. Tension change in response 10^{-7} logM of a cholinergic agonist (carbamoylcholine chloride; Sigma-Aldrich) was recorded using Power Lab (AD Instruments, Hastings, United Kingdom) and normalized to cross sectional area (CSA). CSA was calculated as follows: CSA (mm^2) = tissue wet weight (mg)/ [(length of tissue (mm) x density (mg/mm3)] where the density of tissue was assumed to be 1.05 mg/mm^3.

Intestinal permeability: Mice were exposed to 10 minutes of WAS prior to euthanization. Permeability studies were completed using an *ex vivo* Ussing chamber method. Briefly, 1cm sections of transverse colon and distal ileum were removed and placed in krebs buffer (composition as before). The tissues were cut along the mesenteric boarder, and mounted in an Ussing chamber with an opening of 0.6 cm^2 (World Precision Instruments, Sarasota, Florida, USA). Tissues were bathed with Krebs buffer, oxygenated (95% O_2-5% CO_2) and kept at a constant temperature (37 ºC). The serosal buffer also contained 10 mM glucose as an energy source, and was osmotically balanced by 10 mM mannitol in the mucosal buffer. The chambers contained two voltage-sensitive electrodes (EKV; World Precision Instruments) to monitor the potential difference (PD) across the tissue, and two Ag-AgCl current passing electrodes (EKC; World Precision Instruments). The latter inject the required short-circuit current (Isc) to maintain a zero potential difference as registered via an automated voltage/current clamp (EVC4000; World Precision Instruments). Tissue conductance (G) was calculated by Ohmøs law using the PD and Isc values. Tissues were allowed to equilibrate for 15-25 min before baseline

values for PD, Isc, and G were recorded. Mucosal to serosal flux of the small inert probe (360 Da) [^{51}CR]-EDTA was used to assess paracellular permeability. After equilibration, time zero samples were taken from the serosal buffer, then 6µCi/ml [^{51}CR]-EDTA was added to the mucosal compartment. A maximum radioactive sample was immediately taken from the mucosal buffer, followed by subsequent samples taken every 30 minutes from the serosal buffer (up to 2 hours). Samples were counted in a liquid scintillation counter **(**Beckman Coulter Canada, Inc, Missisauga, Ontario, Canada**)**. Counts from each 30 minute sample were averaged and compared to the maximum radioactive sample. Data were expressed as % flux/cm^2/hr. Results were interpreted as follows: baseline values for I_{sc} indicate ion secretion (i.e. ion movement by active transport); tissue conductance (G) indicates tissue viability; EDTA flux represents macromolecule movement via the paracellular pathway, which can indicate changes in epithelial tight junctions[161].

Inflammation in OBx: Sections of colon and MLN were removed upon euthanization, placed in a sterile microtube, and immediately frozen in liquid nitrogen. Tissue was stored at -80°C until homogenization in Tris HCl buffer containing protease inhibitors (Sigma-Aldrich). Cytokine levels in the supernatant were measured using a BD Cytometric Bead Array (mouse inflammation kit; BD Biosciences, Mississauga, Ontario, Canada) as per the manufactures instructions. Results were analyzed using BD FACSArray Bioanalyzer System (BD Bioscience).

T lymphocytes in OBx mice: Cells were isolated from the spleen and mesenteric lymph nodes using a 10 minute digestion with liberase C1 (Roche, Laval, Quebec, Canada),

followed by disaggregation of the organs using a 40μm cell strainer (BD Biosciences). Fluorescence-activated cell sorting (FACS) was used to identify specific T cell subsets from a heterogeneous population of cells. Briefly, the cells were stained for surface markers to determine the ratio of $CD4^{+}$ T_{H} cells: total lymphocytes. The relative proportion of T regulatory cells was also determined ($CD4^{+}$ $Foxp3^{+}CD25^{+}$). Results were analyzed using BD FACSArray Bioanalyzer System (BD Bioscience).

Neural Activation : Mice were exposed to 10 minutes of WAS prior to euthanization. Tissues were frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek) and stored at -40°C. Distal colons were sectioned to 10 m and mounted onto doublefrost slides (Surgipath, Winnipeg, Canada) and stored at -20°C until processing. Briefly, slides were thawed at room temperature and rinsed in PBS (pH 7.4) to remove residual embedding compound and then fixed by incubating in a 10% buffered formalin solution (VWR, Pennsylvania, USA) for 20 minutes, followed by several washes in PBS. Endogenous proteins were blocked by incubating slides in a 4% normal goat serum block with 0.4% Triton-X (Sigma-Aldrich). Antigen was then detected using an overnight incubation with monoclonal rabbit anti-c-fos antibody (1ug/mL, Cell Signalling, MA, USA). Primary antibody was detected using an Alexa Fluor 488 goat anti-rabbit secondary antibody (1:200, Abcam, Massachusetts, USA) for 2 hours. Slides were then washed in PBS and coverslipped with aqueous mounting medium (Vector Labs, Burlington, Ontario Canada). Images from stained sections were captured using an Olympus BX51 fluorescence microscope (Olympus, Mississauga, Ontario, Canada), and QImaging micropublisher 3.3

RTV camera (QImaging, Surey, BC, Canada). Images were analyzed using Image Pro 6.3 (Media Cybernetics, Bethesda, Maryland, USA) and ImageJ computer software (National Institutes of Health, Bethesda, Maryland, USA). Briefly, threshold limits were set to detect and quantify only c-fos positive fluorescent staining. Positive staining as a percentage of total tissue area was measured using ImageJ measurement analysis.

Serotonin: Mice were exposed to 10 minutes of WAS prior to euthenization. Sections of colon were removed, placed in a sterile microtube, and immediately frozen in liquid nitrogen. Tissues were homogenized in acetic acid and centrifuged. Serotonin levels in the supernatant were assessed using a commercially available ELISA kit (Rocky Mountain Diagnostics, Colorado Springs, Colorado, USA).

Data analysis: Specific analysis of DGGE results was described above. All other experiments were tested using GraphPad Prism (GraphPad software, La Jolla, California, USA). Comparisons were made using unpaired Student's t-tests. A modified t-test was used for data sets that displayed significantly different variances (Welch correction), and non-parametric test were used for data with non-Gaussian distributions. The significance level was set at α=0.05.

Results

Acute stress increases levels of a sympathetic neurotransmitter in the periphery

Exposure to 60 minutes of water avoidance stress resulted in increased levels of the sympathetic neurotransmitter norepinephrine. Higher levels of NE were seen in the serum (1.34 ± 0.2 vs. 4.6 ng/mg ± 1.6, P=0.10) (figure 1A) and whole colon tissue following stress. Only the measurement from the colon reached statistical significance (0.93 ± 0.1 vs.1.37 ng/mg ± 0.1, P<0.05) (figure 1B); however, it is possible that the lack of significance for serum was a type ii statistical error, which could be possibly be corrected by increasing the sample size.

C. rodentium infection in stressed mice

Mice were exposed to 10 days of WAS to examine the effect of host stress on *C. rodentium* colitis. The number of bacteria recovered from the stool of infected mice was similar between stressed (WAS) and non-stressed mice (controls) throughout the first two weeks of infection (figure 2). During the final stages of infection, stressed mice had lower numbers of recovered bacteria, and cleared the *C. rodentium* earlier than their non-stressed controls. Bacteria could not be detected in the WAS group after day 17 post-infection.

Inflammatory responses to the pathogen were examined at two time points during the infection: day 11 (approximate peak colonization of bacteria) and day 18 (approximate peak host adaptive immune response). Analysis of tissue cytokines revealed a significant inflammatory response to the *C. rodentium* compared to uninfected controls.

The addition of WAS resulted in a mild decrease in the levels of all of the proinflammatory cytokines measured compared to non-stressed infected mice (figure 3); however, only IL-6 reached statistical significant (25.1 ± 6.3 vs. 9.3 pg/mg ± 2.0; $P<0.05$) (figure 3A). Again, these results may suffer from a type ii statistical error due to limited sample size. WAS alone did not affect the level of inflammatory markers in the colon.

Work in our laboratory has shown that mice infected with *C. rodentium* exhibit gut dysfunction up to 5 weeks post-infection (unpublished data). The addition of WAS, and the mild reduction in the host inflammatory response, did not have an effect on PI-contractility measures at this time point (figure 4). The model was operational, as there was a significant hypocontractile response seen in the longitudinal smooth muscle of infected and stressed mice (Cr + WAS) vs. uninfected stressed controls (WAS) at the top two carbachol doses ($P<0.05$).

Behaviour in OBx mice

Multiple behavioural tests showed significant changes following olfactory bulbectomy in female mice. Open field activity is the most common measure used in the OBx model. In agreement with the literature, OBx mice showed a significant increase in the total distance travelled (1317 ± 109.9 vs. 2395 cm ± 164.7, $P<0.05$) (figure 5A) and rearing counts (42.3 ± 5.1 vs. 155.3 ± 9.6, $P<0.05$) (figure 5B). In relation to the total distance, OBx mice travelled a significantly lower percent in the anxiogenic center zone (26.2 ± 2.2 vs.12.6% of total ± 0.9, $P<0.05$) (figure 5C). Average velocity did not differ between the two groups, suggesting that locomotor disturbances do not account for the

difference between the groups (33.0 ± 1.0 vs. 33.2 mm/second ± 0.8, ns) (figure 5D). Results from the tail suspension test indicate an increase in despair-like behaviour in OBx mice, as indicated by less time spent trying to escape from an unavoidable stressor (i.e. tail entrapment) (84.9 ± 13.7 vs. 129.8 seconds ± 10.0, $P<0.05$) (figure 5E). Finally, OBx mice show increases in exploratory behaviour with a significantly shorter latency to step-down onto a novel platform compared to sham operated controls (61.1 ± 12.7 vs. 11.49 seconds ± 2.8, $P<0.05$) (Figure5F).

CRH expression is increased in the hypothalamus of OBx mice

RNA was isolated from the paraventricular nucleus of the hypothalamus (figure 6B), which is the primary area of CRH production in the CNS. OBx mice show significantly higher mRNA expression levels compared to sham operated controls (0.72 ± 0.1 vs. 1.58 ± 0.3 normalized fold expression, $P<0.05$) (figure 6A).

OBx mice display post-surgical intestinal dysbiosis

Examination of the dominant bacteria in the stool of OBx and sham mice was completed at 8 weeks post-surgery using molecular methods (PCR-DGGE). Cluster analysis of the banding patterns revealed a significant separation between the two groups that was due to a change in the proportion of certain bacterial populations (figure 7). Altered abundance, rather than presence/absence, accounted for the difference since analysis based on Pearson's correlation coefficient, which is curve based (i.e. accounts for the volume of the band), divided the two groups. In contrast, secondary cluster analysis

using the dice coefficient, which indicates binary variation (i.e. presence/absence) did not clearly separate the two surgical groups (data not shown). The similarity matrix for this data indicated a 60.4% and 60.9% similarity within the sham and OBx groups respectively, and 49.1% similarity between the two groups. Permutation test revealed a significant difference between the two profiles ($P<0.05$).

Gastrointestinal physiology

Fecal output in response to a mild stressor (10 minutes of WAS) was increased in OBx mice compared to sham operated controls (4.3 ± 0.4 vs. 6.7 ± 0.6 pellets, $P<0.05$) (figure 8A). Additionally, the force generated by colonic longitudinal smooth muscle in response to a cholinergic agonist was greater in OBx mice (0.12 ± 0.008 vs.0.24 mg/mm^2 $\pm$ 0.04, $P<0.05$) (figure 8B). Together, these results suggest that colonic motility is increased in OBx mice.

In contrast, there was no significant difference in multiple markers of intestinal permeability when groups of OBx and sham mice were compared. There was no difference in ion transport (Isc) (figure 9A), tissue integrity [conductance (G)] (figure 9B), or paracellular permeability (macromolecular flux) (figure 9C). Both small intestine (distal ileum) and colon were examined.

Inflammatory milieu

Baseline production of inflammatory cytokines was assessed by ELISA. Comparison of OBx and sham mice did not reveal any significant differences in levels of

the pro-inflammatory cytokines IL-1β, TNF-α, IL-6, or IL-17, MCP-1, or anti-inflammatory IL-10 in MLN or colon tissue at week 8 post-surgery (table1). Measurement of myeloperoxidase enzyme activity, a marker of granulocyte infiltration, was below the level of detection (data not shown). Thus, signals related to a variety of different immune system components were not changed in this model, including: T_{H17} and T_{H1} cell signaling (IL-1β, TNF-α); chemotaxis of macrophages (MCP-1); induction of acute phase proteins, differentiation and activity of lymphocytes (IL-6); and neutrophil activity (MPO).

Furthermore, FACS analysis of lymphocytes isolated from both spleen and MLN failed to reveal a change in the percentage of cells expressing CD4+ or cell surface markers associated with T regulatory cells (figure 10).

Neural activation and 5-HT levels in the colon are increased in OBx mice

C-fos is an indirect marker of neural activation. Immunohistochemisty revealed higher levels of the c-fos protein in tissues from OBx mice after exposure to a mild stressor (10 minutes WAS) (0.21 ± 0.06 vs. 0.94 ± 0.20 % area, $P<0.05$) (figure 11A). In addition, colon tissue from these mice had higher levels of serotonin; a modulator of colonic motility (10.7 ± 1.7 vs. 20.1 pg/mg ± 3.0, $P<0.05$) (figure 11B).

Tables and Figures

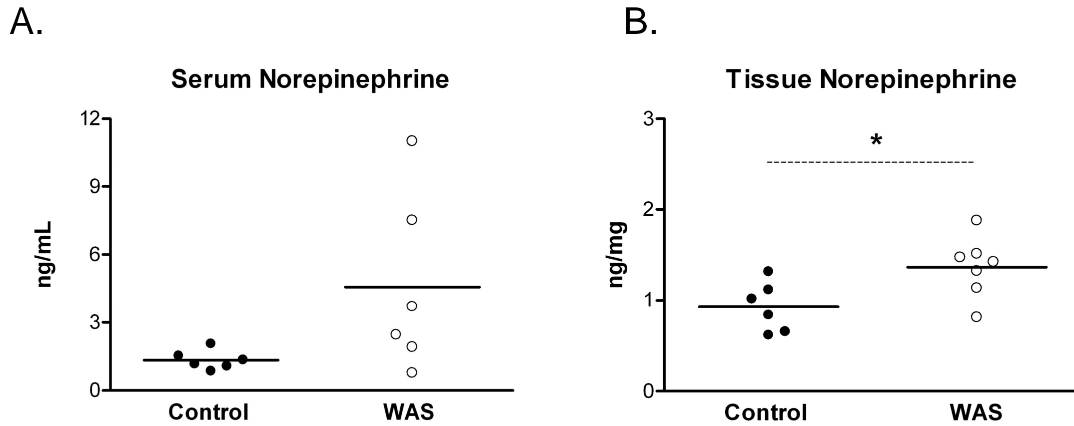

Figure 1. Stress increases levels of a sympathetic neurotransmitter in the periphery. Norepinephrine (NE) levels following 60 minutes of water avoidance stress (WAS). A. Increases in serum NE did not reach statistical significance: P=0.10 (t-test, Welchøs correction). B. NE was significantly increased in the distal colon of stressed mice. * P<0.05.

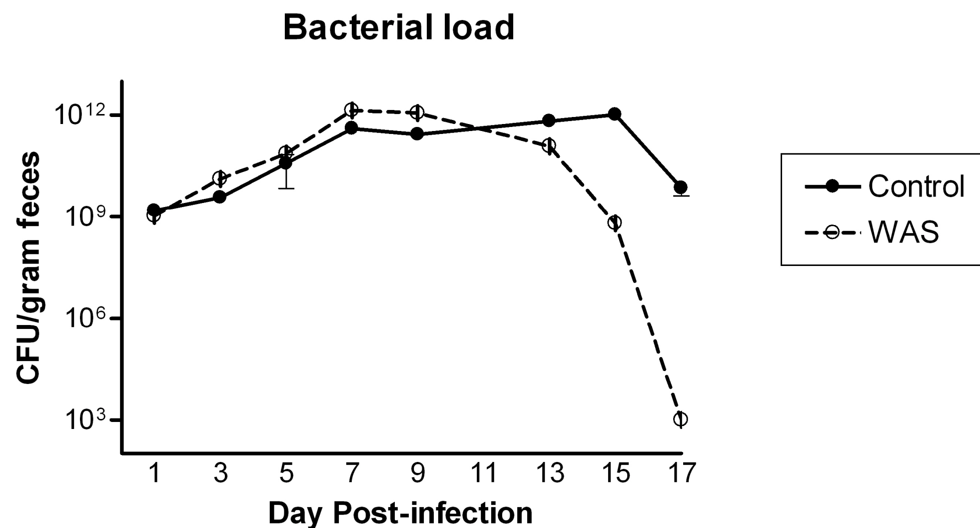

Figure 2. Stressed mice cleared pathogenic bacteria earlier. Levels of *C. rodentium* recovered from the stool of stressed (10 days WAS) and unstressed mice (control) were similar throughout the first two weeks of infection. During the final stage of infection, stressed mice exhibited lower levels of the pathogenic bacteria, and cleared earlier vs. their non-stressed controls.

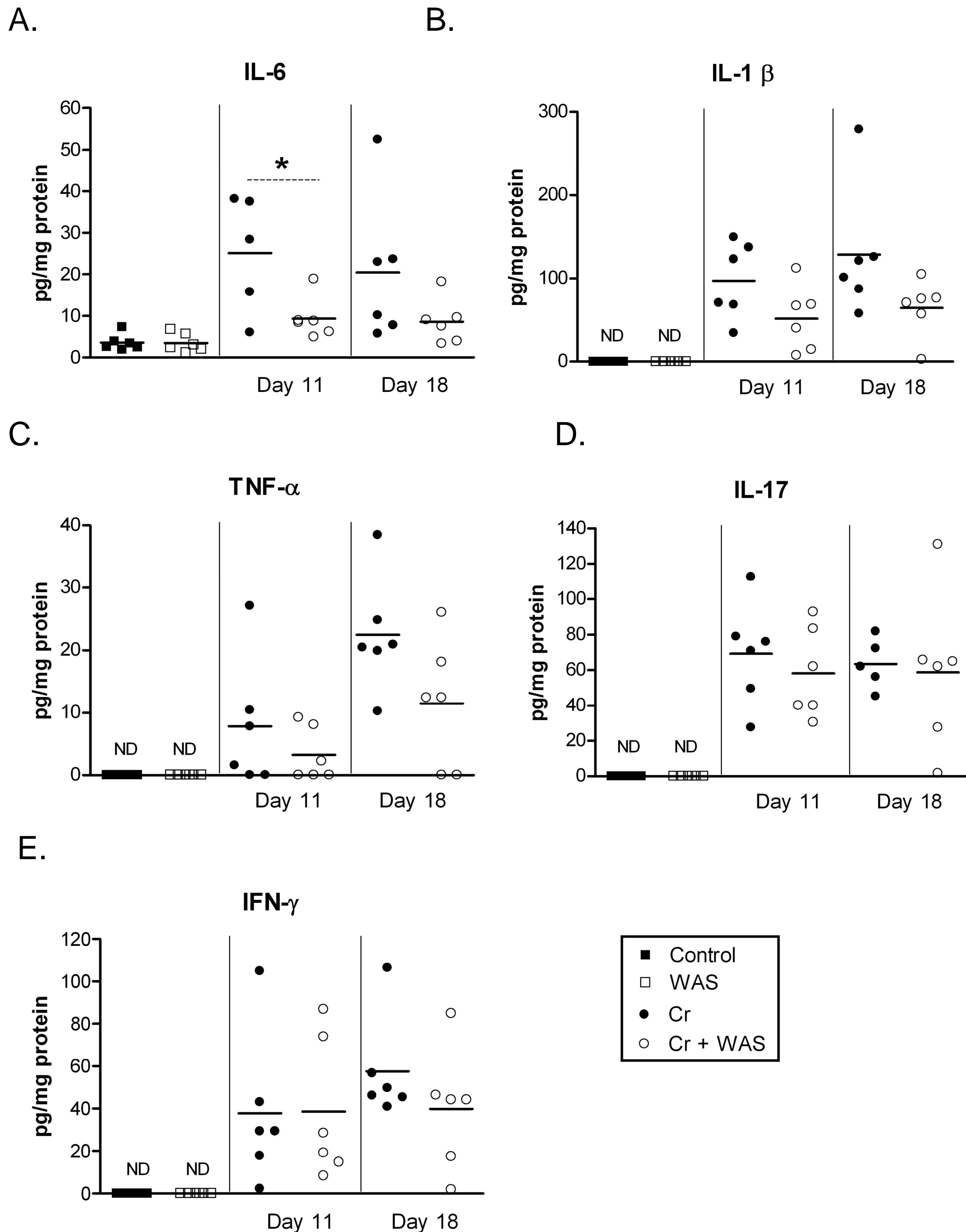

Figure 3. Stressed mice have a blunted inflammatory response to *C. rodentium (*Cr*)*. (A-E) Ten days of WAS resulted in lower levels of pro-inflammatory cytokines in the colon of mice with *C. rodentium*-induced colitis; however, only IL-6 reached statistical significance. Two endpoints were measured: day 11 (peak of infection), day 18 (peak of colitis). * $P<0.05$. ND= none detected.

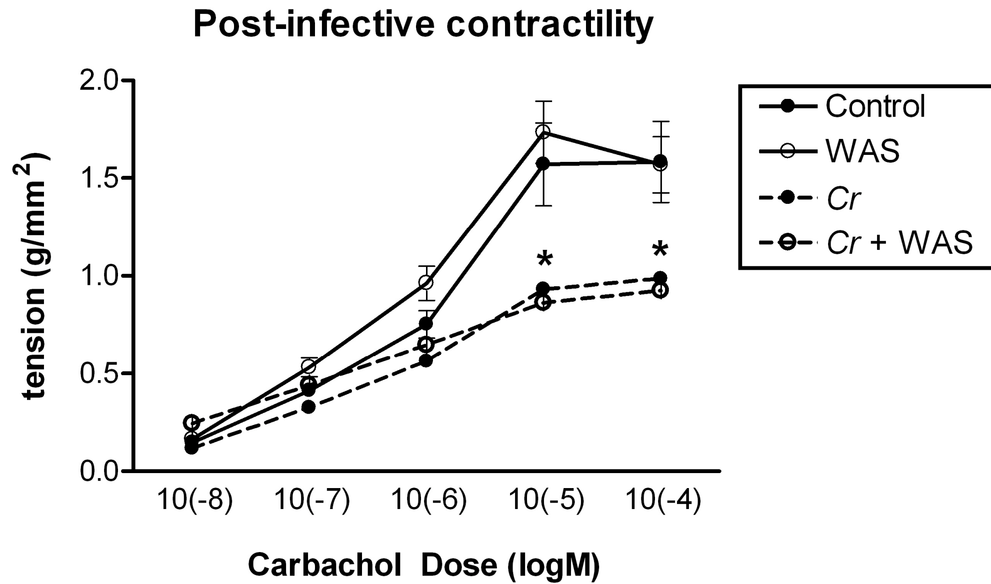

Figure 4. Stress does not worsen post-infective gut dysfunction. Colonic longitudinal smooth muscle from infected mice shows a significant hypocontractile response when stimulated with a cholinergic agonist 5 weeks post-infection. The addition of 10 days of stress did not have a significant effect on post-infective dysfunction. * $P<0.05$ (Cr + WAS) vs. WAS at doses logM 10^{-4} and logM 10^{-5}. Control (n=2); WAS (n=6); *Cr* (n=6); *Cr* + WAS (n=6).

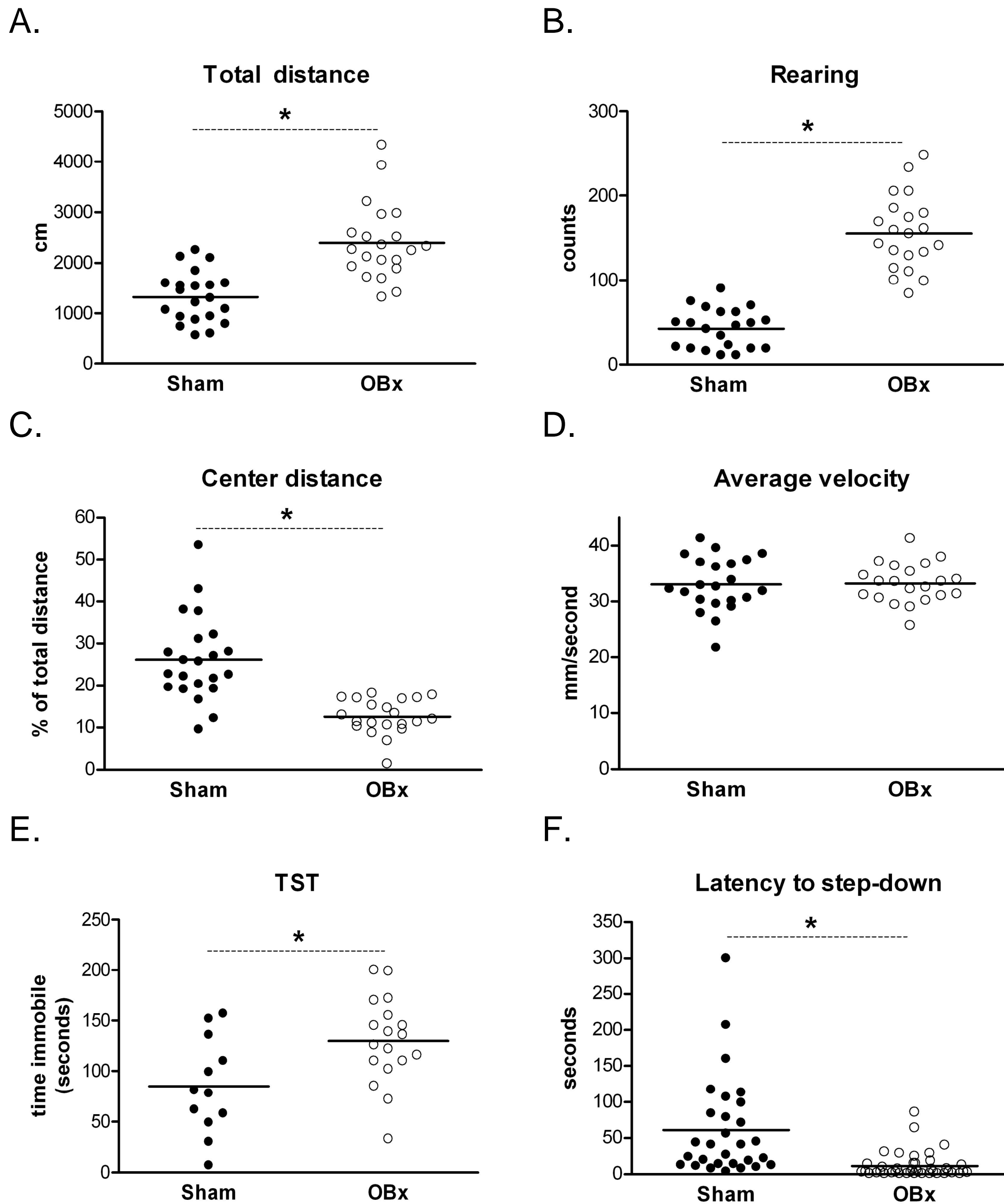

Figure 5. OBx mice demonstrate significant behavioural changes. (A-D) open field behaviour. A. female mice exhibit hyperlocomotion in an open field; a hallmark trait of the model. Mice also show increased rearing (B) and decreased distance in the axiogenic center zone (C). No significant difference was seen in average velocity (D). E. Mice show a significant increase in the duration of immobility in the tail-suspension test. F. Mice show decreased latency to step-down. * $P<0.05$.

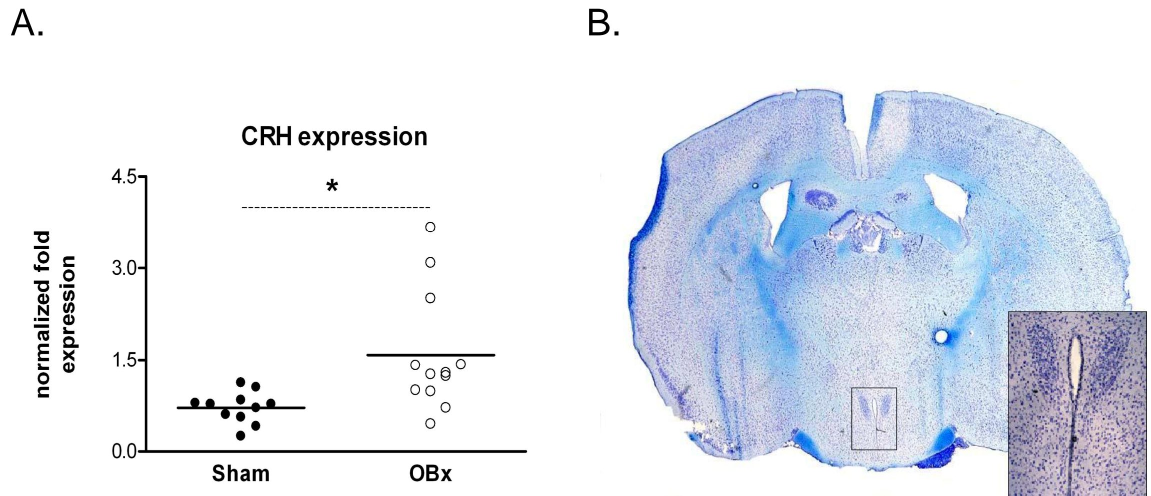

Figure 6. OBx mice have increased CRH expression in the hypothalamus. A. mice have significantly higher levels of CRH mRNA in the PNV of the hypothalamus at 8 weeks post-surgery. * $P<0.05$. B. Coronal brain section at the level of the PVN stained with toluidine blue. Black box and inset picture (lower right) indicates the area that was collected for RNA isolation.

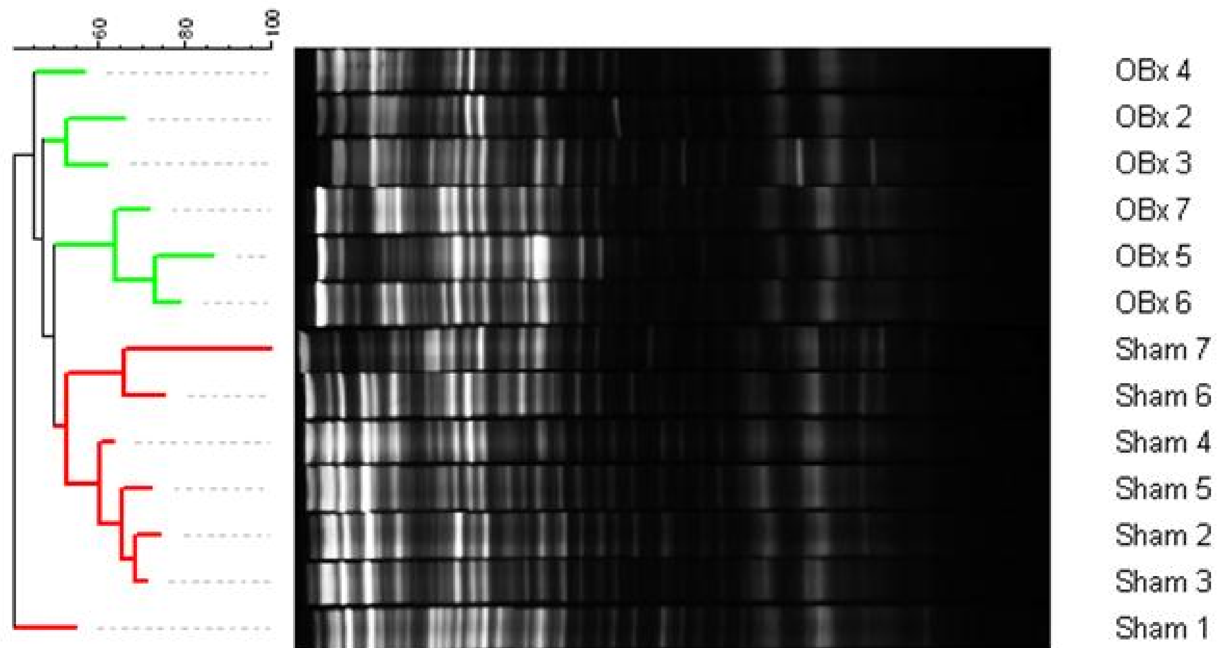

Figure 7. OBx mice display post-surgical intestinal dysbiosis. Cluster analysis of the fecal microbiota at 8 weeks post-surgery shows a clear separation between OBx and sham mice. The similarity matrix for this data indicated a 60.4% and 60.9% similarity within the sham and OBx groups respectively, and 49.1% similarity between the two groups.

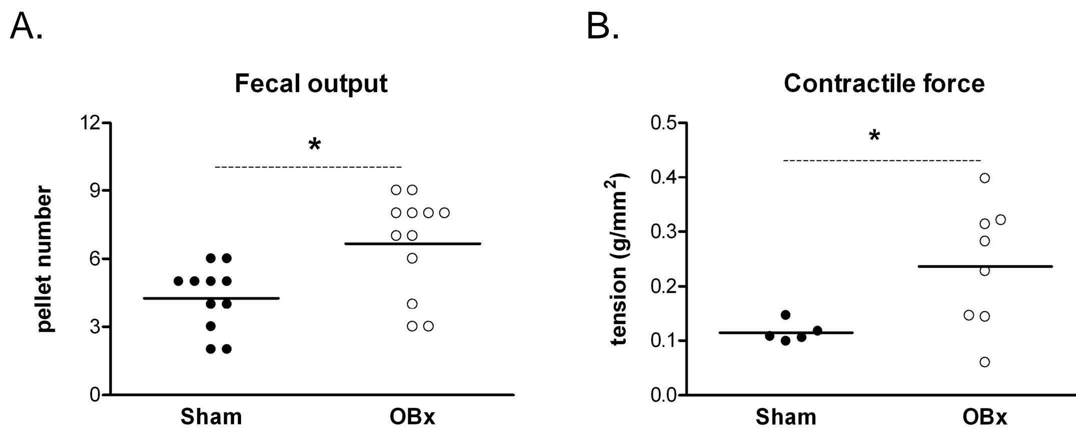

Figure 8. Colonic motility is increased in OBx mice. A. mice show increased fecal output in response to a mild stressor (10 minutes WAS). B. Longitudinal smooth muscle from the colon of OBx generated significantly greater contractile force in response to a cholinergic agonist vs. sham operated controls (carbachol, dose logM 10^{-7}). * $P<0.05$.

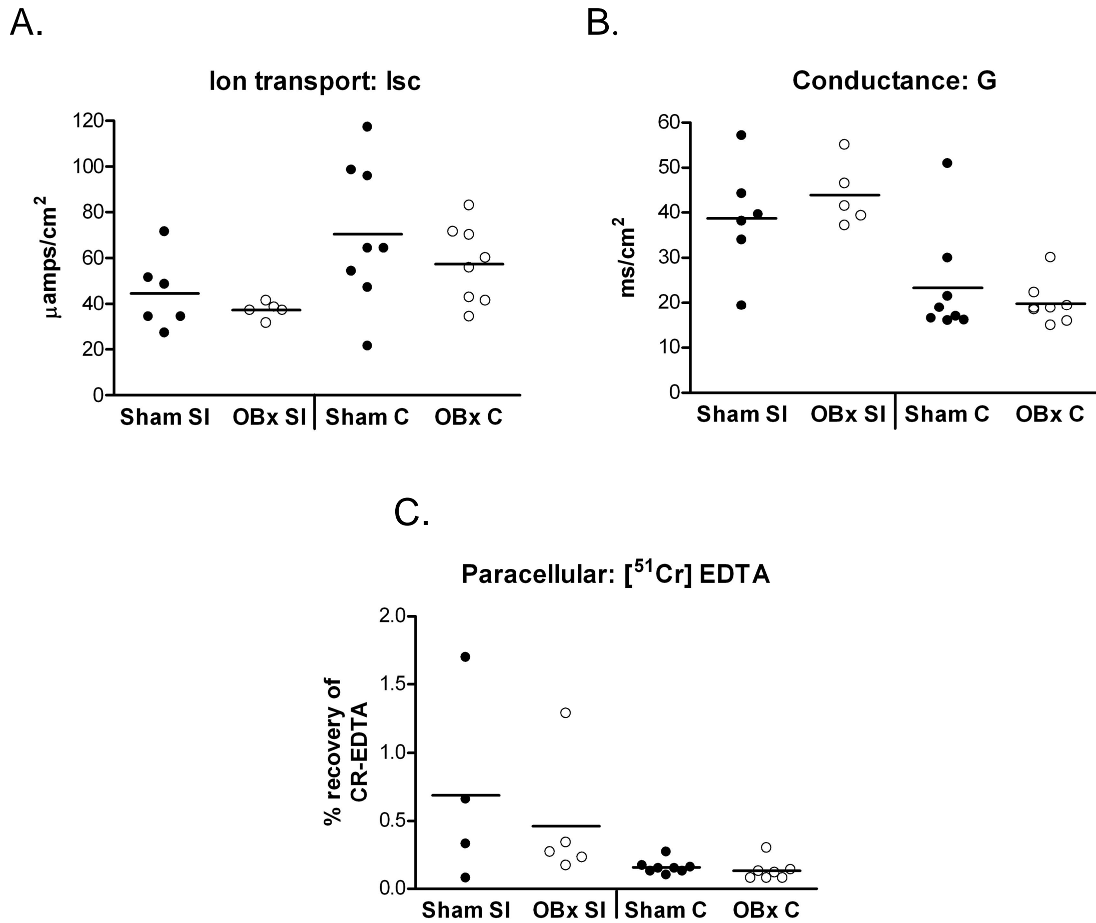

Figure 9. OBx does not affect GI permeability. Ussing chamber studies did not reveal any significant changes in parameters of intestinal permeability in the distal small intestine (subgroup SI, distal ileum) or the colon (subgroup C) of bulbectomized mice. Parameters examined were: A. short circuit current (I_{sc}); B. tissue conductance (G); and C. paracellular flux of radio labeled EDTA.

Table 1. OBx does not affect markers of inflammation in the gut. Measurements from mesenteric lymph nodes (MLN) and whole colon tissue did not reveal any significant changes in unstimulated levels of inflammatory cytokines in OBx mice at 8 weeks post-surgery. ND= not determined; SEM= standard error of the mean.

	Sham MLN		OBx MLN		Sham colon		OBx colon	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
IL-1β	**12.20**	±1.67	**10.97**	±0.63	**2.39**	±0.30	**2.33**	±0.53
TNF-α	**4.90**	±0.50	**5.95**	±0.40	**1.74**	±0.12	**1.74**	±0.38
IL-6	**18.21**	±1.84	**16.58**	±1.75	**5.72**	±0.46	**5.49**	±0.79
IL-17	**12.13**	±1.38	**13.65**	±1.54	**3.24**	±0.23	**2.45**	±0.40
MCP-1	ND		ND		**0.13**	±0.01	**0.15**	±0.04
IL-10	**0.43**	±0.15	**1.05**	±0.32	**0.35**	±0.10	**0.48**	±0.12

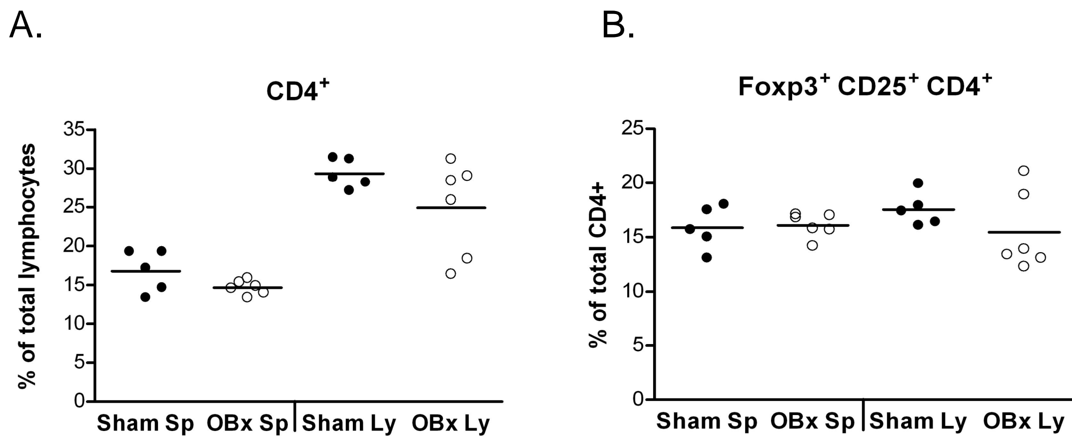

Figure 10. OBx does not affect T helper or T regulatory cell balance. A. OBx did not affect the percentage of lymphocytes expressing $CD4^+$ surface markers in either the spleen (subgroup Sp) or the mesenteric lymph nodes (subgroup Ly) at 8 weeks post-surgery. B. The percentage of $CD4^+$ cells that were positive for surface markers indicative of T regulatory cells ($CD25^+$ $Foxp3^+$) was also unchanged.

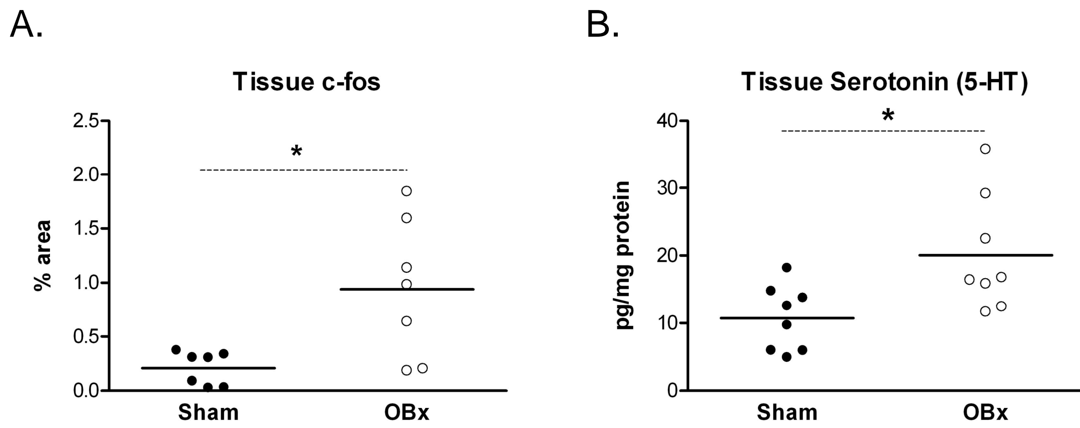

Figure 11. OBx mice have increased neural activation and 5-HT levels in the colon. A. OBx mice have higher levels of c-fos protein in the colon after exposure to a mild stressor (10 minutes WAS). B. Assessment of serotonin levels revealed a significant increase in this pro-motility neurotransmitter in the colon as well. *$P<0.05$

Discussion

This work exemplifies the undeniable influence of host stress on the immediate response of the gastrointestinal tract and adaptation of the microbiota within. It demonstrates central and peripheral changes in a model of anxiety co-morbid depression, which provide a novel mechanistic description of stress-induced dysbiosis. Individual aspects of the chronic model are well supported by the literature; however, this works unifies separate components of a complex relationship between the brain and the gut.

Initially, we demonstrated that moderate levels of psychological stress could influence components of the stress response in the periphery by showing WAS-induced increases in NE in the colon. While information regarding stressed-induced NE in the GI tract is sparse due to technical limitations, this observation is supported by a study by Alverdy *et al.*, which used high performance liquid chromatography to show a significant increase in NE concentration in the cecal contents of surgically stressed mice [32]. Since we were able to increase the NE content in the colon, we wanted to test the idea that host stress may worsen an infection with an AE pathogen via NE-induced increases in virulence. This theory tied together two independent risk factors for the development of PI-IBS: antecedent psychological stress and severity of the initial infection. This interaction had been shown *in vitro* [162], but had yet to be shown *in vivo* in an animal model of psychological stress. Consequently, we exposed mice to one hour sessions of WAS during the first 10 days of a *C. rodentium* infection. In contrast to the expected

outcome, we saw a mild decrease in the host inflammatory response to the pathogen, and earlier clearance of the bacteria from the stressed mice. We attributed the lower cytokine levels to a general dampening of the immune response by stress-induced glucocorticoids and catecholamines. This mild decrease in inflammation did not affect measure of PI-gut dysfunction (i.e. colonic contractility). Alternatively, it is possible that the lack of cultured bacteria at the later time points, which was attributed to earlier clearance, was actually due to greater retention of the pathogen. However, culturing of the ceacum and its contents post-mortem did not reveal any *C. rodentium*, and thus argues against this possibility (data not shown). Furthermore, while *C. rodentium* is a murine model of EHEC and EPEC, the existence the adrenergic receptor seen in *E. coli* [163] has not been specifically shown in *C. rodentium*. It is possible that these two pathogens, which share mechanisms of lesion formation, may have different quorum sensing (QS) systems. Indeed, in 2007, Coulthurst *et al*. published a report that demonstrated a different QS system, involving *N*-acylhomoserine lactone, in *C. rodentium* that is not present in *E. coli* [164]. Thus, we decided that this was not the ideal model system to study the effects of host stress on susceptibility to GI dysfunction and disease. Additionally, it became apparent that disadvantageous host responses may require chronicity, and that a better understanding of the baseline consequence of stress exposure were required.

Olfactory bulbectomy provided a model of chronic activation of the stress response [125, 128] in adult mice that did not require daily exposure to experimental stressors, drug treatment, or environmental manipulation. Additionally, it was strongly supported

by literature that purported it to be a well-rounded representation of patients with anxiety co-morbid depression, with both predictive and face validity. Most of the OBx work had been completed in rats, however, reports suggested that mice shared many of the characteristics of the model [119, 120, 122, 165]. Consequently, we believed it would be a well suited model to study the influence of chronic host stress on the GI tract. Since verified adrenergic QS is confined to a minority of enteric pathogens, we decided to switch our focus to the resident microflora, with the notion that you must first understand the community before a pathogen is introduced.

Initial work with the model involved verification of anxiety- and despair-like behaviour as well as a heightened stress response following surgery. The hallmark behavioural trait in OBx is hyperactivity in an open-field. It has been suggested that this feature is due a lack of behavioural dysinhibition, which is controlled by the amygdala prior to surgery [114]. In other words, the mice are unable to habituate to the new surroundings, and therefore continually perceive them as novel and anxiogenic, resulting in increased exploration and movement. In accordance with the literature, we consistently observed increased activity in the OBx mice as indicated by increased total distance, and rearing. Although rare, mice that failed to travel a greater distance than their paired control were excluded from further studies. OBx mice also travelled more in the outer boarders of the arena vs. the center. This behaviour is related to a conflict between the innate tendency to explore vs. tactile safety of the outer walls, and has been noted in OBx mice before [165]. A test that is purported to be based on a similar principle, the step-down

test, showed opposite results, i.e. the OBx mice step down onto the novel surface quicker. This is similar to the conflicting results gathered by Song and Wang in the T-maze [117, 118]. In this specific instance, the step-down results for the OBx mice could be interpreted as an increase in exploratory behaviour, which is supported by previous results in the novel object test [165]. Alternatively, it may simply be due to their hyperactivity and agitated state. Further examination of exploratory behaviour in these mice could confirm or contest this finding. Finally, we used the tail suspension test to assess despair-like behaviour. This test is generally used to screen drugs with potential anti-depressive efficacy; however, it has been used to show induction of despair-like behaviour in a chemical model of depression [153]. In accordance, we showed an increased duration of immobility in OBx mice compared to sham operated controls. This group of behavioural tests validates the use of OBx as a model of anxiety comorbid depression in our hands.

We had shown that OBx mice exhibited heightened emotionality in a mildly stressful environment. Next, we determined if there was increased activation of the central stress response. We chose to measure basal, rather than stress-induced CRH expression, because it would provide stronger evidence of persistent modulation of the stress response, and thus support the notion that the model represented chronic psychological perturbation. Our measurements showed a significant increase in CRH mRNA expression in OBx mice when the PVN was specifically isolated. Previously, others had failed to show an increase in CRH mRNA levels in the external layer of median eminence of the hypothalamus in male rats [130]. However, our method isolated the

primary area of CRH production (the PVN) and used a highly sensitive method of quantification (real-time PCR). These results verify chronic activation of the central stress response in OBx mice as great as 2 months post-surgery, and substantiate OBx as a model of persistent activation of the stress response.

As previously mentioned, microbiota play an integral function in host defense via pathogen displacement, production of antimicrobial factors, induction of protective immunoglobulins, and maturation and maintenance of the mucosal immune system [47]. Hence, alteration of community structure may alter host susceptibility to disease. Accordingly, we studied the effect of persistent host stress on the community structure of the colonic bacteria. Since the microbiota of OBx mice had not been previously examined, we used DGGE methods in order to capture an overall impression of the microflora. Analysis was done at 8-10 weeks to allow sufficient time for the chronic effects of stress to be established. Furthermore, initial qualitative screening in this model indicted that changes did not begin to appear until after 6-8 weeks post-surgery. Cluster analysis of the profiles from OBx and sham mice repeatedly showed distinct visual separation in dendrograms created using the Pearson's correlation coefficient and the neighbour-joining method [155, 156, 158]. Further analysis using randomization tests showed that the generated differences (i.e. the randomly shuffled data) varied significantly from the real (i.e. experimental) differences, indicating that the treatment have a significant effect . Visual inspection, as well as the comparison of results obtained using the dice coefficient (band based) [155] vs. the Pearson's coefficient (curve based) [156], suggest that the

differences stem from modification to the abundance of certain groups of bacteria, rather than their presence or absence. These findings are in line with the literature for both IBS and stress-related dysbiosis in humans, which repeatedly show increases and decreases of different groups of bacteria at the phylum level. A few studies have shown unique species in IBS [166]; however, these results were obtained with targeted qPCR for opportunistic pathogens, and may be exclusive to this study population. Importantly, environmental and behavioural-induced changes to the flora are not likely to play a role in this model. Surgical groups were kept in the same cage (i.e. OBx and sham have the same environmental exposure) and reports from the literature suggest that OBx does not result in chronic changes to feeding behaviour [167, 168]. Further characterization of the microbiota in OBx using molecular sequencing based approaches (e.g. Pyrosequencing) will elucidate the specific bacteria that are responsible for the divergence in the profiles in these mice. Ultimately, these results show that there is a shift in the community in the colon of mice in the presence of chronic activation of the stress response. This provides a unique model of stress-induced dysbiosis in adult mice that does not rely on repeated exposure to environmental stressors.

While stress-induced dysbiosis has been shown in other models, the underlying mechanisms have not been established [29, 108-113]. We examined two physiological parameters in GI tract that are significantly modulated by stress in both clinical and animal studies, and that could alter the habitat of commensal bacteria: gut-motility and -permeability. Since the control of global motility is complex and based on the net result

of a variety of stimulatory and inhibitory influences, we first used a simple test of overall motility. Fecal output has been used as an indicator of gastrointestinal motility [159], as well as stress-responsiveness [169]. We utilized the method to assess GI response to a mild stressor in OBx mice, who demonstrated an increased fecal output during 10 minutes of WAS. Since expulsion of fecal matter is largely controlled by the colon, we decided to complement this finding with *in vitro* studies of colonic contractility using the organ bath method. While the data showed significant variability in both controls and OBx, the contractile force of the longitudinal muscle was consistently greater in OBx compared to sham operated controls. Significant differences were found at a carbachol dose of logM 10^{-7}, suggesting that freshly excised tissue from OBx mice was hyper responsive to physiological doses of a cholinergic agonist. OBx also showed an increased response to pharmacological doses of carbachol (logM 10^{-5} , logM 10^{-4}), but these differences were not significant. The results from both of these experiments suggest that OBx results in a moderate increase in colonic motility that is most pronounced during periods of stress.

In contrast, we did not see a significant difference in measures of barrier integrity and paracellular permeability. All parameters measured showed a high level of concordance between the two groups, suggesting that the intestinal barrier is not affected by chronic stress activation. While this finding is in disagreement with many studies that show stress-induced changes in permeability, none of the previous studies have been completed with this level of chronicity of stress. Studies claiming to study the effects of chronic stress have used 15 days of crowding stress [170], or 12 days of CRH

administration[95]. It is possible that adaptations have occurred over time, resulting in normalization of permeability in our model. Prolonged activation of the stress response can lead to decreased CORT release; a factor that was shown by Meddings and Swain to be important for stress-induced changes to permeability [97]. It is also possible that the changes are more subtle and not captured by the parameters studied. For example, transcellular permeability to macromolecules such as horseradish peroxidase (HRP) was not examined; nor was expression of tight junction proteins. A more detailed inspection of barrier function, and earlier time points would help clarify the issue. Furthermore, it seems that CRH-induced changes in motility and permeability may have different mechanisms, so the presence of one change may not ensure the other. Evidence to support this notion comes from animal studies that show differing effects of adrenalectomy on CRH-induced motility and permeability, with the former being unaffected, and the latter being abolished. Also, the majority of studies analyzing permeability use peripheral administration of CRH, whereas most motility studies utilize central administration. Thus, the site of action and the effector molecule that produce stress-induced changes may be not be the same. Lastly, motility is altered in a region-dependent manner (i.e. decreased in stomach, increased in colon), whereas stress-induced increases in permeability are universal. This suggest a greater role for central neural-mediated control of stress-induced dysmotility, which varies along the GI tract (i.e. greater vagal innervations in the more proximal regions), and perhaps more peripheral endocrine-mediated control of permeability. Further agonist and antagonist studies using different routs of administration are needed to determine if this is correct.

Having established OBx-induced changes in motility, we next examined possible mechanisms to explain this observation. Previous work in our laboratory has shown that pro-inflammatory cytokines could modulate the release of neurotransmitters from the enteric nervous system [171, 172], and that cytokines and T cell levels influence *in vitro* dysmotility induced by enteric infection [173, 174]. For this reason, we examined the inflammatory response in OBx mice compared to sham operated controls. We found no significant difference in any of the cytokines or chemokines measured, and furthermore, we did not find any difference in the ratio of $CD4^+$ T cells or T regularly cells at 8 weeks post-surgery. This was somewhat unexpected as various reports in the literature showed immune alterations in OBx mice. There are several factors that could explain this discrepancy. First, many of the measurements in the literature are taken less than a month after surgery [118, 121], consequently, post-surgical sequel could be present in their animals, or adaptation could have occurred in ours. Also, the vast majority of the reported modifications are revealed under immune stimulation, and are measured in the periphery [116, 121, 146] . For example, Connor *et al.* reported that peripheral levels of IL-1β and TNF-α were different in OBx and sham rats in response to an *in vivo* challenge with lipopolysaccharide[146]. Thus, results examining unstimulated levels of inflammatory markers in the colon and MLN may not be comparable. We specifically wanted to assess baseline cytokine quantities since these would be the physiological levels in the OBx mice on a chronic basis. While stimulated levels may be more relevant in disease models, we were more interested in stress-induced changes in the absence of overt inflammatory

challenge, as well as those that occurred specifically in the gut. Thus GI inflammation does not appear to play a role in OBx-induced gut dysfunction and associated dysbiosis.

Next, we examined neural mechanism within the gut. This approach seemed rational considering that CRH-induced changes in motility seem to have a strong neural basis. Our first assessment used c-fos as a marker of neural activation following acute stress (10 minutes WAS)[175]. Tissues from OBx mice showed significantly greater binding of the c-fos antibody as indicated by greater density of staining. The staining was localized to the myenteric plexus, suggesting that stress results in greater activation of the ENS in OBx mice compared to sham. Next, we assessed levels of serotonin in the colon after this same stress protocol. Serotonin, in conjunction with ACh[85], has been suggested as one of the final modulators in CRH-induced dysmotility, as the effects of centrally-administered CRH can be block by peripheral injection of 5-HT_3 receptor antagonist [176]. In accordance with these data, we found a significantly greater amount of serotonin in the colon tissue of OBx mice following stress exposure.

Taken together, these results suggest that OBx causes increased activation of the central stress response, resulting in intestinal dysmotility and concomitant bacterial dysbiosis. The dysmotility appears to be mediated by an increased parasympathetic outflow to the enteric nervous system, and subsequent increases in levels of the pro-kinetic neurotransmitter serotonin. These results provide for the first time a plausible mechanism to explain stress-induced dysbiosis in an animal model. Furthermore,

similarities between the OBx model and some patients with IBS suggest that these factors may contribute to microbial community disruption in some subsets of the disorder.

In summary, this thesis shows the influence of both acute and chronic host stress on the GI tract. Findings suggest that chronic periods of stress exposure are required for aberrant GI responses and the expression of disease. This work provides further support for the role of microbiota in stress-related GI disorders and provides a mechanistic description of stress-induced dysbiosis. Future studies related to this topic that will further contribute to the field, and promote translation of this knowledge, should focus on inhibition of stress-induced changes in both GI physiology and microbiota using both central (i.e. CNS-targeted) and peripheral (i.e. ENS-targeted) pharmacological approaches.

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