Modulation of the Brain-Gut-Microbiota Axis in a Murine Model of Inflammatory Bowel Disease

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Biology

by

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Professor Melanie Gareau

2014
The Thesis of Jacob Raymond Emge is approved and is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2014
DEDICATION

I dedicate this thesis to my family –
My mother, for teaching me to laugh
My father, for teaching me to work
My brother, for teaching me to do both simultaneously

And to Tina Lanquist, for sparking my passion in biology
that I’ll forever carry with me
EPIGRAPH

*Be fearful of mediocrity.*
Jonathan Ellery
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ABSTRACT OF THE THESIS

Modulation of the Brain-Gut-Microbiota Axis in a Murine Model of Inflammatory Bowel Disease

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Jacob Raymond Emge

Master of Science in Biology
University of California, San Diego, 2014

Professor Kim E. Barrett, Chair
Professor Lorraine Pillus, Co-Chair

Anxiety, depression, and altered memory are increasingly being associated with intestinal diseases, including inflammatory bowel disease (IBD). Understanding the link between these behavioral changes and IBD may provide important clinical relevance since concomitant mood disorders often increase a patient’s risk of requiring surgery
and developing secondary functional gastrointestinal diseases (FGIDs).

In the current study, anxiety-like behavior, as assessed by the light/dark box test, and non-spatial memory, as assessed by the novel object test, were determined at the peak and following the resolution of inflammation using the dextran sodium sulfate (DSS) mouse model of acute colitis. DSS was administered for 5 days via drinking water followed by either 3 or 9 days of normal drinking water for comparing behavior during active versus resolved inflammation, respectively. Mice were weighed throughout the study, colon lengths were measured at the time of sacrifice, and histological analysis was conducted in order to assess the degree of colonic disease. In addition, the composition of the gut microbiota was characterized using qPCR of DNA extracted from fecal pellets.

Mice at 8 days post-DSS demonstrated impairments in non-spatial memory and anxiety-like behavior compared to controls. These behavioral defects did not persist following resolution of intestinal inflammation and were normalized by 14 days post-DSS. Furthermore, shifts in the composition of the gut microbiota were evident at the peak of intestinal inflammation – notably as decreases in lactobacilli and segmented filamentous bacteria (SFB) – which were also reversed by the time of resolution.

Taken together, our findings support the hypothesis that in a murine model of IBD, changes in mood and behavior are present during acute inflammation in the presence of shifts in the composition of the gut microbiota.
I.

INTRODUCTION
It is now well known that the brain can communicate with the gastrointestinal (GI) tract in a complex, bidirectional relationship known as the brain-gut axis. Through a series of parallel systems of nerves, hormones, cytokines, etc., various regions of the brain and the organs of the GI tract are in constant communication with one another, establishing an intricate network of both positive and negative feedback loops. With this knowledge, it is not surprising that shifts in mood and emotions, ranging from fear to love, are often associated with sensations of knots or butterflies in the gut. Although these feelings are metaphorical in nature, they are rooted in biology and are prime examples of the brain influencing intestinal physiology. Increasingly, awareness is emerging regarding intestinal physiology influencing the brain, affecting mood and behavior. Our goals are to assess how inflammation within the gut can lead to changes in cognitive function and behavior.

1.1 Brain-Gut Axis

The brain-gut axis is modulated by a collection of pathways with each one playing a unique role in the overall communication between the brain and the intestinal tract. For top-down influence, the brain utilizes the parasympathetic and the sympathetic branches of the autonomic nervous system, a variety of monoaminergic pathways, as well as the hypothalamic-pituitary-adrenal (HPA) axis, which regulates stress responses. For communication in the opposite direction, the intestinal tract conveys sensory information to the central nervous system (CNS) via afferent neurons, enteroendocrine cells, and mucosal immune cells. Together, this network can allow for a complex communication system.
Primary sacral and vagal afferent neurons, with terminals that lie along the intestinal tract, play a critical role in transmitting sensory information from the periphery to the CNS. These afferent neurons are responsible for conveying mechanical stimuli, whether due to normal gut physiology or noxious changes, directly to either the spinal cord or brain stem (Mayer, 2011). In addition, signaling molecules, including hormones and neurotransmitters, released by other intestinal cells may stimulate these afferent neurons. For instance, mucosal immune cells respond to chemical changes in luminal contents by releasing signaling molecules (Mayer, 2011). These molecules, such as serotonin or acetylcholine, bind to receptors on nearby afferent neurons (Barbara et al., 2007), allowing mucosal immune cells to convey information directly to the nervous system. In addition, enteroendocrine cells along the intestinal tract respond to shifts in luminal content through the release of hormones and paracrine signals, which also have receptors on nearby afferent neurons (Mayer, 2011). A recent study demonstrated that a microbial shift within the colon can stimulate cholecystokinin secretion from enteroendocrine cells (Leslie et al., 2003), providing an example as to how shifts in the composition of the gut microbiota are conveyed to the CNS.

In the context of inflammatory bowel disease (IBD), studies have demonstrated that molecules related to inflammation are able to stimulate primary afferent neurons. When interleukin-1 beta (IL-1β) is administered to rats intravenously, vagal afferents are activated (Ek et al., 1998).

An important modulator of brain-to-gut communication is the stress response sensed by the HPA axis. The HPA axis is composed of three major hormone-releasing glands – the hypothalamus, the anterior pituitary, and the adrenal cortex. The HPA axis is
stimulated by either physical or perceived psychological stress, with the degree of stimulation dependent on the intensity and duration of the stressor (Koolhaas et al., 2011). The immediate effect is the release of corticotropin-releasing factor (CRF) from the hypothalamus, which triggers the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which, in turn, stimulates the secretion of cortisol (humans)/corticosterone (rodents) from the adrenal cortex. Glucocorticoid receptors at both the hypothalamus and anterior pituitary, serve an inhibitory effect, establishing the HPA axis as a negative feedback loop (Reul et al., 1985). Assessment of serum corticosterone levels can be used as a quantitative indication of whether mouse models are stressed as a result of experimentation.

1.2 Inflammatory bowel disease (IBD)

IBD is an idiopathic condition characterized by chronic relapsing and remitting of inflammation along the GI tract. There are two major subsets of IBD – Crohn’s disease (CD) and ulcerative colitis (UC), which are distinguished by the type of inflammation and its distribution along the length of the GI tract. In UC, the inflammation is mucosal, continuous, and restricted to the colon. In CD, the inflammation is transmural, and it can occur in discontinuous patches anywhere along the GI tract (Laass et al., 2014).

Symptoms of IBD differ amongst the 4 million patients living with CD or UC across Europe and the United States (Martin-Villa, 2014) and include fatigue, fever, chronic diarrhea, weight loss, and abdominal pain (Mawdsley et al., 2006). IBD is commonly accompanied by a variety of conditions outside of the GI tract, ranging from skin ulcers to bile duct inflammation (Marzano et al., 2014). In addition, patients
suffering from IBD also have an increased risk of developing colorectal cancer although this has begun to decrease within the past decade. (Castaño-Milla et al., 2014) Despite all of this knowledge, the exact etiology of IBD remains unclear. However, four main factors are thought to contribute to the condition – microbial imbalance in the gut (intestinal dysbiosis), overt immune responses, genetic susceptibility, and unknown environmental triggers. These factors can partly be mimicked using mouse models of disease, which can recapitulate some, but not all, patient symptoms of IBD.

1.3 IBD mouse models

There are currently a variety of well-established murine models of IBD, with each one characterized by a different type of inflammation either in its pathology or distribution. These include chemical models, genetic models, and hapten-induced models. The selection of an appropriate model for study is therefore very important.

Dinitrobenzenesulfonic acid (DNBS) is an effective colonic irritant used to induce UC-like inflammation when administered via intracolonic installation (Cuzzocrea et al., 2001), with inflammatory pathology resolving by 6-weeks post administration. Inflammation in this model can be reinitiated in the presence of stress along with additional DNBS administration but not by DNBS alone (Qiu et al., 1999). This stress-induced inflammation may indicate the sensitivity of this model’s inflammatory activity to brain-gut axis manipulation.

Oxazolone administered rectally results in a colitis-like inflammation restricted to the terminal half of the colon. In regions experiencing inflammation, mucosal immune cells of the T helper type 2 (Th2) lineage show increased production of interleukin-4 (IL-
4) and transforming growth factor-beta (TGFβ). In the oxazolone model, the administration of anti-IL4 improves colonic conditions (Boirivant et al., 1998).

When administered intra-rectally, 2,4,6-trinitrobenzenesulfonic acid (TNBS) in ethanol results in a colonic inflammation that is characteristic of UC. Macrophages permeate mucosal and sub-mucosal layers during active inflammation (Tozawa et al., 2003). In addition, TNBS-induced inflammation is characterized by an imbalance in T-helper cell cytokine expression (Tozawa et al., 2003).

Although these models are well established and used frequently, they all require researchers to administer chemicals through the rectum (Boirivant et al., 1998; Tozawa et al., 2003), potentially serving as a stressor for mice. Therefore, to avert this confounding variable, an increasingly more common murine model of IBD is the dextran sodium sulfate (DSS) model (Ding et al., 2014; Gresnigt et al., 2014; Ohkusa et al., 1985; Yuge et al., 2014) in which the mucosal irritant is administered via a drinking water solution in either an acute or a chronic setting. DSS administration results in pathology similar to UC as well as diarrhea, bloody stools, mucosal ulcers, and weight loss (Cooper et al., 1993; Okayasu et al., 1990). The DSS mouse model of colitis is preferable in studying the brain-gut-microbiota axis because it lacks the stressful means of administration required by other chemical models of IBD.

1.4 IBD and mood disorders

IBD, both CD and UC, have a strong association with psychiatric illness in patients suffering from active or resolved intestinal inflammation (Filipovic et al., 2014). The exact mechanism by which these behaviors and IBD correlate with one another is
currently unclear, and the evidence is somewhat ambiguous. Regardless of the current controversy, the basic relationship between IBD and mood disorders is well supported through case studies, basic research, and clinical analysis.

Beginning the late 1980s, studies emerged demonstrating the correlation between psychiatric illness and IBD. In one study amongst 162 patients suffering from either active or remissive UC or CD, subjects were evaluated through a series of questionnaires to assess their degree of anxiety and depression. IBD patients demonstrated an increase in both of these behaviors in comparison to controls, with patients experiencing active CD demonstrating psychiatric illness at a higher rate than patients in remission (Andrews et al., 1987). Such an affiliation between active and resolved UC was not demonstrated (Andrews et al., 1987). In a more recent longitudinal study, CD patients were assessed over a two-year period in an attempt to correlate these psychiatric conditions to the activity of their disease as indicated by the Crohn’s Disease Activity Index (CDAI). Researchers found a weak association between feelings of hopelessness and anxiety to higher CDAIs and a strong positive correlation between depression and CD activity (Mardini et al., 2004).

The association between IBD and psychiatric co-morbidities is becoming clearer as research continues to support the notion that the two are correlated by a bidirectional pathway through which not only physical illness dictates mood, but mood also affects disease. For instance, IBD patients suffering from psychiatric illness have a decreased chance of entering remission compared to those that have no psychiatric symptoms; 17% of IBD patients recover from illness when psychiatrically ill while 53% of patients will enter remission when psychiatrically well (Andrews et al., 1987).
In addition to these psychiatric dispositions, voluntary behaviors can also affect disease severity. In a study in which disease activity was monitored over a three-year period, patients who had adopted behaviors to help them cope with stress demonstrated less severe disease (Küchenhoff, 1995). Patients demonstrating behaviors of a depressed individual showed a more severe illness over a longer period of time (Küchenhoff, 1995; Stasi et al., 2008). With stress having this correlation to illness, it is reasonable to speculate that stress and its perception may also provide a link between IBD and behavior. In rodent models, chronic stress has been proven to increase the permeability of the intestinal tract and to sustain intestinal inflammation (Meddings et al., 2000). HPA-axis activation may therefore provide a readout for brain-gut communication in mouse models of IBD.

Placing mechanism aside, the stress of a patient suffering from any chronic disease would reasonably be increased and therefore be a potential key contributor to the overall condition. However, this is not the case. In a study conducted in the early 1980s, CD patients were assessed on the following three behaviors: depression, obsessive-compulsion, and phobia. Overall, a much higher prevalence of these behaviors was demonstrated in CD patients in comparison to the control group, which was made-up of individuals each suffering from one of a variety of chronic medical conditions (Helzer et al., 1984), demonstrating that psychiatric co-morbidities in IBD patients are not a result of sickness behavior.

In mouse models of disease, using DSS to induce chronic colitis, anxiety-like behavior was demonstrated through the use of the light/dark box test (Bercik et al., 2010). This change in behavior was attributed to a mechanism involving the vagus nerve since
these changes in behavior were not demonstrated in vagotomized mice exposed to chronic DSS administration (Bercik et al., 2010). Despite these findings, behavior in acute DSS colitis has not been assessed.

1.5 Gut microbiota

Ninety percent of the cells that make-up the human body are non-human cells of prokaryotic origin. The majority of these species reside in the large intestine in the magnitude of $10^{12}$-$10^{14}$ colony forming units (CFU) per colon. These 100 trillion microorganisms represent over forty thousand species and millions of genes not represented in the human genome (Kurokawa et al., 2007). The role of these bacteria, commonly referred to as the gut microbiota, is not fully understood. However, evidence continues to emerge demonstrating the importance of these species to the metabolism of the host and, more recently, to the psychiatric state of the host.

In order to evaluate the metabolic importance of gut symbionts, researchers have begun conducting experiments demonstrating how shifts in the composition of the gut microbiota can result in changes in host metabolism. When microbes, including *Bifidobacterium breve*, a beneficial probiotic organism, were orally administered to mice, researchers found an altered profile of fatty acid composition in adipose tissue in comparison to controls that were not administered with microbes (Wall et al., 2009). Colonizing germ-free (GF) mice, which are completely axenic and devoid of microbiota, with a normal gut microbiota caused mice to undergo a 60% increase in total body fat regardless of a reduction in the amount of food consumed (Bäckhed et al., 2004). In a study demonstrating the duality of this phenomenon, researchers transplanted the fecal
microbiota from lean mice or their obese twins into GF mice. After the transplant, the mice receiving fecal microbiota from an obese mouse increased in body and fat mass. However, this increase was prevented when both groups of mice having undergone a fecal transplant were co-housed (Ridaura et al., 2013). Therefore, subjecting mice to the same environment promotes normalization of the gut microbiota.

Although they are not completely understood, microbes of the gut play a critical role as symbionts within their human host. Therefore, undesirable shifts in the composition of these species may be responsible for a variety of human diseases, and conversely, transplanting fecal microbiota from healthy donors may be a useful treatment in both chronic and acute conditions such as *C. difficile* infection, diabetes, and IBD (Collins et al., 2013).

### 1.6 Brain-gut-microbiota axis

In recent years, attention has been brought to a role for the gut microbiota in the context of the brain-gut axis in a dynamic that is newly being coined as the brain-gut-microbiota axis. Researchers have demonstrated that the bacteria residing within the GI tract can play a role beyond their established metabolic and digestive functions. Evidence has shown that shifts in the composition of these bacteria can precipitate changes in host’s mood and behavior. Although the exact means by which they cause this effect is unclear, physiological components critical to the mechanism have been – and continue to be – identified.

Early studies of the brain-gut-microbiota axis demonstrated that infection with the bacterial pathogen *Campylobacter jejuni* corresponds with increased neuronal
activity, as indicated by c-fos expression in the vagal ganglia and in the visceral sensory nuclei in the brainstem (Goehler et al., 2005). C. jejuni was also demonstrated to induce anxiety-like behavior in mice as exhibited by the hole-board test (Goehler et al., 2008), which correlated with the degree of c-fos expression in the bed nucleus of the stria terminalis (Goehler et al., 2008), suggesting that elements of this microbial infection is conveyed to the brain. Taken together, these findings suggest a direct route of communication through which a shift in the composition of the gut microbiota is conveyed to and throughout the CNS, leading to changes in behavior.

Impairment in behavior due to altered gut microbiota composition is not necessarily permanent as it may be improved by normalizing the composition of the gut microbiota (Clarke et al., 2013). Similarly, in the DSS mouse model of chronic colitis, anxiety-like behavior can be normalized by treatment with Bifidobacterium longum in mice with a functioning vagus nerve (Bercik et al., 2011a).

Non-human primate studies demonstrated that psychiatric disposition can also alter the gut microbiota. For instance, when infant rhesus monkeys are subjected to early life stress via maternal separation, the gut microbiota of the infant is disrupted as demonstrated by a decrease in lactobacilli (Bailey et al., 1999). This model also results in altered gut physiology in the infant, suggesting that the gut is playing a key role in the communication pathway. In rodent studies, following exposure to maternal separation, intestinal barrier function is altered, and there is an increase in visceral sensitivity (O’Mahony et al., 2011).

In addition to baseline changes, some pathogenic infections demonstrate psychiatric changes only in the presence of a second stimulus such as an exposure to
stress. In studies from our group, mice infected with the bacterial pathogen *Citrobacter rodentium* showed memory dysfunction but only when following exposure to acute psychological stress (Gareau et al., 2011). This same study showed that memory impairment can be reversed by normalizing the composition of the gut microbiota with the treatment of *Lactobacillus*-containing probiotics (Gareau et al., 2011), drawing a correlation between gut microbiota dysbiosis and behavior. In contrast to specific-pathogen-free (SPF) controls infected with *C. rodentium*, GF mice demonstrated baseline changes in non-spatial memory, which were not altered following exposure to acute psychological stress (Gareau et al., 2011). GF mice also show decreased levels of anxiety-like behavior in comparison to wild-type controls (Clarke et al., 2013) as well as increased tryptophan concentration in blood – both of which are normalized by colonizing the GF mice with normal gut microbiota (Clarke et al., 2013). Interestingly, gut bacteria may serve as a factor more significant than genetics when determining behavioral phenotype. When GF BALB/c mice are colonized with the gut microbiota from the innately more exploratory NIH Swiss mice, their exploratory behavior increases to that of NIH Swiss mice (Bercik et al., 2011b). Conversely, when GF NIH Swiss mice are colonized with the gut microbiota from normal BALB/c mice, their exploratory behavior is reduced as demonstrated by the light preference test (Bercik et al., 2011b). These studies highlight the important nature of the brain-gut-microbiota axis in modulating behavior.
Understanding the brain-gut-microbiota axis in the context of IBD may elucidate the foundation of the behavioral abnormalities seen in patients, providing potential strategies for future therapies.
II.

MATERIALS AND METHODS
4.1 Animals

Male and female C57BL/6 mice (6-8 weeks of age) were used for all experimental groups. Mice were housed in cages lined with chip bedding with free access to food and water. Animals were kept at a UCSD animal facility, and behavioral testing was performed in a biosafety cabinet. All procedures and protocols were reviewed and approved by IACUC at the University of California, San Diego.

4.2 Novel object test

After a one-hour habituation in individual cages, mice underwent the novel object test composed of three phases in the following sequence: training, resting, and testing.

Training phase

A smooth napkin ring (Object #1) and a star-shaped cookie cutter (Object #2) were placed in opposite corners of the cage (Sarkisyan et al., 2009). The mice were given 5 minutes to investigate the objects while under video surveillance. At the conclusion of the training phase, objects were removed, and mice were allotted 20 minutes as a resting phase in the same cage. The training phase confirmed that the mice had an equal preference for Object #1 and Object #2.

Testing phase

Following the resting phase, Object #2 (referred to as Object #2b during the testing phase) was reintroduced to the cage along with a new object, Object #3, in
opposite corners of the cage (Sarkisyan et al., 2009). Mice were allotted 5 minutes to investigate the new object (#3) and the old object (#2b) under video surveillance.

The videos were later analyzed for the number of times the mice approached or sniffed each object. Results were expressed as an exploration ratio, which was calculated using the following equation: \[
\frac{\text{frequency of smell #3}}{\text{frequency of smell #3 + frequency of smell #2b}} \times 100.
\]
This calculation indicated the mouse’s preference for either of the two objects (Mumby et al., 2002): A value of 50% indicates that the mouse investigated both objects equally while a higher value indicated that the mouse investigated the new object more than the old object (Plescia et al., 2014). The mouse was considered to investigate an object if its nose came within 2 centimeters of it.

4.3 Light/dark box test

In order to measure anxiety-like behavior, mice were placed in a light/dark box for 10 minutes, and behavior was video recorded for analysis. Approximately one-third of the box is dark while the other two-thirds is kept well lit. Mice demonstrating a higher degree of anxiety-like behavior spend more time in the dark portion of the box in comparison to less anxious counterparts. The total amount of time a mouse spent in the light box, reflecting anxiety, and the frequency of transitions, reflecting activity, between the two portions was assessed (Mumby et al., 2002).

4.4 Study design

At 6-8 weeks of age, experimental mice were provided a 3% DSS drinking water solution as the only source of water that was made available ad libitum from day 1 to day
5. At day 5, the DSS drinking water solution was removed and untreated drinking water was provided for the remainder of the study. Control mice were given untreated drinking water throughout the study and weighed daily. Mice were tested for behavior, and samples were collected at either 8 days or 14 days post-DSS, corresponding to active disease or resolution of colonic inflammation, respectively.

On the day of testing, mice were transferred in their original cages to a biosafety cabinet and allowed to acclimatize for 60 minutes. During acclimatization, mice remained in their original cage within a biosafety cabinet. After acclimatization, mice were subjected to the light/dark box test. Immediately after completion of light/dark box testing, mice were placed in clean individual cages and allowed to habituate for 60 minutes. During habituation, mice were placed in individual cages within a biosafety cabinet. After habituation, mice underwent the novel object test. Immediately after completion of behavioral testing, mice were sacrificed by CO₂ inhalation and cervical dislocation. At the time of sacrifice, fecal contents were collected and stored at -80°C in order to perform qPCR on bacterial DNA at a later date. Blood was collected via cardiac puncture, centrifuged at 5000 RPM for 10 minutes, and stored at -80°C in order to measure serum corticosterone levels at a later date.

### 4.5 Corticosterone

To measure corticosterone levels in blood serum, a commercial enzyme immune assay (EIA) kit (Enzo Life Sciences) was utilized, and samples were quantified using a fluorescent plate reader (Victor V, Perkin Elmer). The results were presented as ng/ml.
4.6 Histology

Distal colons were collected and fixed in 10% formalin. Samples were embedded in paraffin, and 5 µm sections were cut onto polarized glass slides. Sections were processed for H&E staining.

4.7 qPCR

Colonic fecal samples were collected at sacrifice and frozen at -80°C. Bacterial DNA from stool was extracted using a kit (Qiagen) while following the manufacturer’s instructions. Isolated DNA was amplified via qPCR using SYBR and primer sets (Table 1), targeting the 16S rRNA gene of bacterial species (Barman et al., 2008; Petnicki-Ocwieja et al., 2009), including Eubacteria (all bacteria; housekeeping gene), E. rectale, SFB, Bacillus, Lactobacillus, Enterobacteriaceae, Bacteroides, and Firmicutes to compare overall colonization patterns between DSS and control groups. These primers were previously validated to exclude cross reactivity (Barman et al., 2008; Petnicki-Ocwieja et al., 2009). The qPCR conditions consisted of: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C followed by 1 minute at 60°C. A melt curve was used to ensure quality control. Results are presented as percentage expression of each species relative to total Eubacteria.

4.8 Statistics

Results are expressed as a means +/- standard error (SE). The p-values were calculated using the two-way Student’s t-test or ANOVA followed by a Neuman-Keuls post-hoc test as appropriate using GraphPad (San Diego, CA).
Illustration 1. Novel object test. During the training phase, each mouse is introduced to Object #1 and Object #2 for 5 minutes (A). During the testing phase, each mouse is allotted 5 minutes to investigate Object #2b and Object #3 (B). The number of times the mouse investigates each object is recorded.
Illustration 2. Light/dark box test. Mice are placed individually in the light/dark box for 10 minutes. The amount of time each mouse spends in the lit portion of the box is recorded as well as the frequency of transitions between the lit and dark portions of the box.
Table 1. Primer sequences employed in analysis of the microbiota (Gareau et al., 2011).

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<td>Firmicutes</td>
<td>GCTGCTAATACCGCATGATATGTC</td>
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III.

RESULTS
3.1 DSS administration results in colitis-like disease

I first sought to establish the model to be used in these studies. As expected, DSS administration in mice via the drinking water (3% mass by volume) resulted in changes in body weight. Peak weight loss was observed by 8 days post-DSS, corresponding to active acute colonic inflammation (Figure 1). Weights began to normalize by 9 days post-DSS and continued to do so until the time of sacrifice at day 14 post-DSS, corresponding to the resolution of inflammation (Figure 1). Correspondingly, by day 8 post-DSS, colon lengths were significantly shorter in the DSS group in comparison to controls (Figure 2). This change persisted when colonic inflammation had resolved at 14 days post-DSS (Figure 2).

Histological analysis at 8 days post-DSS demonstrated colonic inflammation compared to controls (Figure 3). Colons from the DSS group showed a prevalence of neutrophilic infiltration, increased vascularization, and edema (Figure 3C and 3D), which were absent in controls.

3.2 DSS administration results in anxiety-like behavior without affecting exploratory behavior

I next examined whether colitis was associated with any changes in behavior in the mice. Anxiety-like behavior was assessed in mice using a light/dark box and measuring time spent in the light box. DSS-treated mice demonstrated anxiety-like behavior as indicated by a decrease in the total time spent in the light box versus the dark box compared to controls (Figure 4A). This behavior began to normalize and was no longer significantly different at 14 days post-DSS compared to controls (Figure 4A).
The number of times mice transitioned between the light and dark portions of the light/dark box was calculated as an indicator of overall behavior. A decrease in this parameter would reflect sickness behavior, which might have affected the exploratory ratio independent of anxiety. However, the frequency of transitions was not affected by DSS administration neither at the peak nor at the resolution of colonic inflammation compared to controls (Figure 4B).

3.3 DSS administration results in memory impairment

Non-spatial memory was assessed using the novel object test and calculated as the frequency of exploration of a novel versus a known object. DSS-treated mice demonstrated impairment in non-spatial memory in comparison to controls as indicated by a significantly lower exploration ratio (Figure 5). This improvement in non-spatial memory was significantly improved once the colonic inflammation had resolved at 14 days post-DSS, with similar exploration ratios between the DSS and control groups (Figure 5).

3.4 DSS administration does not activate the HPA-axis

In order to assess activation of the HPA-axis, serum corticosterone levels were determined by EIA. Serum corticosterone was not significantly different between DSS and controls neither at 8 nor 14 days post-DSS (Figure 6).
3.5 DSS administration causes shifts in the composition of gut microbiota

The composition of the microbiota was assessed at both 8 and 14 days post-DSS by qPCR for the 16SrRNA gene and compared to control mice. The proportions of *E. rectale*, segmented filamentous bacteria (SFB), *Bacillus*, and *Lactobacillus* (Figure 7A-D) were decreased in the 8 days post-DSS group vs. controls when compared to the universal primer for Eubacteria. The shift in *Lactobacillus* was normalized by 14 days post-DSS (Figure 7D) whereas the shift in SFB was partially reversed (Figure 7B). In contrast, neither the composition of *Bacteroides* nor Enterobacteriaceae was affected by DSS administration (Figure 7E and 7F). Finally, the proportion of Firmicutes was not affected by DSS administration at 8 days post-DSS; however, a significant decrease in Firmicutes occurred by 14 days post-DSS (Figure 7G).
Figure 1. Percent change in body weight. DSS administration resulted in peak weight loss at 8 days post-DSS. Weight began to increase at 9 days post-DSS and continued until 14 days post-DSS. (*p<0.05 compared to the relevant control by Student’s t-test; N=14-16 mice/group.)
Figure 2. **DSS-induced colonic shortening.** DSS administration resulted in colonic shortening, which was not resolved by 14 days post-DSS. (*p<0.05 compared to the relevant control by one-way ANOVA followed by a Newmann-keuls post-hoc test; N=14-16 mice/group from 4 experiments.)
Figure 3. Colon histology. DSS administration resulted in colonic inflammation at 8 days post-DSS as demonstrated by H&E staining. (A and C) scale bar 500 µm. (B and D) scale bar 250 µm.
Figure 4. Light/dark box testing for anxiety-like behavior. DSS administration resulted in anxiety-like behavior at 8 days post-DSS, which was resolved by 14 days post-DSS (A). DSS administration did not affect the number of transitions between the light and dark portions of the light/dark box (B). (*p<0.05 compared to the relevant control by Student’s t-test; N=10-12 mice/group from 4 experiments.)
A.

Time Spent in Light (s)

Control (8d)       DSS (8d)       Control (14d)     DSS (14d)

B.

Frequency of Transitions (#)

Control (8d)       DSS (8d)       Control (14d)     DSS (14d)
**Figure 5. Novel object test for non-spatial memory.** At 8 days post-DSS, the DSS group demonstrated a significant impairment in non-spatial memory in comparison to controls as indicated by a significantly lower exploration ratio. This behavior improved at 14 days post-DSS. (*p<0.05 compared to the relevant control by Student’s t-test; N=10-12 mice/group from 4 experiments.*)
**Figure 6. HPA axis activation.** DSS administration did not impact the concentration of corticosterone in serum at either 8 or 14 days post-DSS. (N=8-15 mice/group from 4 experiments.)
**Figure 7. Alterations in the composition of the gut microbiota.** The proportion of: *E. rectale* (A), SFB (B), *Bacillus* (C), and *Lactobacillus* (D) was significantly decreased 8 days after the administration of DSS compared to controls. The shifts in SFB (B) and *Lactobacillus* (D) were reversed by 14 days post-DSS. Neither Enterobacteriaceae (E) nor *Bacteroides* (F) composition was affected by DSS administration. The proportion of Firmicutes was not affected at 8 days post-DSS; however, a significant decrease in Firmicutes composition occurred by 14 days post-DSS (G). (*p<0.05 compared to the relevant control by Student’s t-test; N=5-13.*
Figure 7. continued
C.  

**Bacillus**

![Bar graph showing the percent of Eubacteria for different groups and time points.](image)

D.  

**Lactobacillus**

![Bar graph showing the percent of Eubacteria for different groups and time points.](image)

Figure 7. continued
E. Enterobacteriaceae

F. Bacteroides

Figure 7. continued
G.

Firmicutes

Figure 7. continued
IV.

DISCUSSION
This study demonstrates that DSS administration resulting in acute colonic inflammation is associated with increased anxiety-like behavior and impairment in non-spatial memory, which normalize by the resolution of inflammation at 14 days post-DSS. As indicated by serum corticosterone levels, administration of DSS does not result in activation of the HPA axis. Notably, these behavioral changes coincide with shifts in the overall composition of the gut microbiota. Taken together, these finding suggest that colonic inflammation results in shifts in the gut microbiota, which, in turn, are correlated with changes in mood and behavior.

Anxiety-like behavior can be assessed in mice using the light/dark box test to measure time spent in the light box (Bourin et al., 2003). Here, DSS administration results in anxiety-like behavior at 8 days post-DSS, as indicated by a decreased time spent in the light box that normalizes by 14 days post-DSS. In accordance with these findings, DSS administration has previously been shown to result in anxiety-like behavior in a chronic model of colitis using the latency to step-down test for anxiety (Bercik et al., 2011a). As an indicator of overall activity levels, the frequency of transitions between the light and dark portions of the box were recorded and compared between the control and experimental groups. With the frequency of transitions between the two groups being nearly equal, this indicated that activity levels were not impaired by colonic disease and weight loss. This supports the notion that the anxiety-like behavior we observed was not simply resulting from sickness behavior in the DSS mice, which would be reflected by decreased overall activity compared to controls. This suggests that the presence of anxiety in a murine model of IBD can mimic similar findings in human patients.
The novel object test can be used to indicate the level of non-spatial memory in mice (Ennaceur et al., 1989) where mice are examined for their ability to remember an old versus a familiar object. Accordingly, DSS administration resulted in non-spatial memory impairment at 8 days post-DSS, which normalized by 14 days post-DSS. Previous studies have shown that shifts in the gut microbiota, as demonstrated by terminal restriction fragment length polymorphism, correlate to altered memory as shown by the Barnes maze (Ohland et al., 2013). Our group has also demonstrated that microbial infection with the murine enteric bacterial pathogen *C. rodentium* results in stress-induced memory impairment as measured by the novel object test (Gareau et al., 2011).

Given that both *C. rodentium* infection and DSS colitis results in colonic inflammation, inflammation may serve as a potential mechanism of action in altered cognitive function in these models.

GI function results from a complex set of interactions both physiological and psychological in nature (Nardone et al., 2014). When stress is perceived in humans, higher centers of the brain will trigger the HPA axis as well as the autonomic nervous system, supporting fight or flight responses (Fink, 2011). Previously, corticosterone administration has been shown to result in memory impairment as indicated by the step-down latency test (Santos et al., 2014). Since the DSS group in this study did not demonstrate altered serum corticosterone levels, the resulting behavioral impairments were not due to HPA axis activation. It would be interesting to note, however, that perhaps exposure to stress could lead to changes at 14 days post-DSS.

Recently, evidence has emerged showing that GF mice demonstrate more anxiety-like behavior than gnotobiotic mice lacking only specific pathogenic bacteria as
demonstrated by the open-field and marble-burying tests (Nishino et al., 2013). In studies involving *C. rodentium* infection, it was demonstrated by our lab that bacterial infection resulted in changes in the composition of the microbiota as determined by qPCR (Gareau et al., 2011). This technique can be used for characterize the overall composition of the intestinal microbiota using known specific primers and comparing them to a universal primer. Using this technique, it was demonstrated that shifts in the composition of the gut microbiota coincide with acute colitis in the DSS model, including *Lactobacillus* and SFB, which are of particular interest in the context of colonic inflammation.

Many species of *Lactobacillus* are classified as probiotic organisms that are thought to be beneficial to the host. In the current study, a decrease in *Lactobacillus* at 8 days post-DSS was observed, which returned to normal by 14 days post-DSS. Many members of the *Lactobacillus* genus are capable of metabolizing lactose to lactic acid and other products. A recent study showed that one product in particular, Calpis sour milk whey, a fermented milk product of *Lactobacillus helveticus*, improves memory in mice as indicated by the novel object test (Ohsawa et al., 2014). Mechanistically, *Lactobacillus* consumption has been shown to increase GABA expression in the brain, an effect not seen in vagotomized mice (Bravo et al., 2011). Consequently, the vagus nerve may serve as an important mechanism for the brain-gut-axis responsible for communicating the underlying shift in microbiota to the brain. Therefore, the decrease in *Lactobacillus* species observed at 8 days post-DSS, but not at 14 days post-DSS, may in part explain the impact on behavior.

Similarly to *Lactobacillus*, a decrease in SFB was also observed at 8 days post-DSS, which reversed with the resolution of inflammation. SFB have recently been shown
to play a primary role in the induction of T helper 17 (Th17) cell responses (Lécuyer et al., 2014), which promote inflammation (Joller et al., 2014). Although SFB may play a critical role in the pathogenesis of colonic inflammation in mouse models (Tanabe, 2013), they are also critical in the maintenance of gut homeostasis since SFB serve a protective role against pathogenic bacteria such as *C. rodentium* (Baker et al., 2012). Therefore, in this study, the decrease in SFB colonization at 8 days may be playing a role in modulating behavior in our DSS mice, with recovery by 14 days post-DSS although a precise mechanism is not currently known.

In future studies, metagenomic analysis is likely to be a useful tool to identify untargeted species of bacteria in the gut microbiota rather than the targeted approach we undertook to assess the composition of specific bacterial species. Elucidating these large- and small-scale shifts in all bacterial species would provide a more precise sense of the changes in the microbial composition that could later be attributed to specific bacteria through the use of more precise techniques such as, for example, genome sequencing. Additional behaviors, including spatial or working memory and depression in DSS colitis may also provide helpful information in assessing the overall impact of colitis on changes in mood and behavior, providing interesting findings.

In conclusion, I have demonstrated that the DSS model of acute colitis leads to changes in behavior, including anxiety-like behavior and cognitive deficits that may result from shifts in the composition of the gut microbiota due to colonic inflammation. Elucidating the gut dysbiosis associated with colitis may provide the key to combating the behavioral comorbidities seen in patients suffering from IBD.
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