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Defining Microbial Signatures of Gut Dysbiosis in Models of Anxiety-related Disorders

Christopher Fields

DEFINING MICROBIAL SIGNATURES OF GUT DYSBIOSIS IN MODELS OF ANXIETY-RELATED DISORDERS

by

CHRISTOPHER FIELDS

Under the Direction of Geert J. de Vries, PhD

ABSTRACT

The gut microbiome is a complex community featuring a bewildering array of microbial species. Over the past couple decades, there has been an explosion of research demonstrating that the gut microbiota plays critical roles in a variety of host functions, including immune modulation, metabolism, brain function, and behavior. Mechanistic approaches such as fecal microbiota transfer from disease models into healthy animals have demonstrated direct effects of gut microbiota on host parameters but sequencing of fecal samples from similar subjects across different cohorts often reveals wide differences in microbial composition. This wide variability is also seen in clinical subjects within specific disease states postulated to be influenced by gut

microbiota. Nevertheless, there are likely core features of the gut microbiota that may be modulated across different disease conditions to transmit similar signals to the host.

In this dissertation, I focus on potential core features of gut dysbiosis, or alterations in gut microbiota associated with various disease states. In Chapter 2, I will explore variations in gut microbiota observed across a genetic model exhibiting varying behavioral profiles, namely Brattleboro rats. In Chapter 3, I explore the potential mechanistic links between gut microbiota and host behavior, using a treatment that compromises the integrity of the gut barrier (namely, adding food emulsifiers to the diet). Compromising the gut barrier allows increased access of microbial byproducts that affect the CNS. I explored this potential mechanism in Chapter 4 by testing the effects of gut-derived LPS on host behavior, as LPS can compromise gut barrier integrity even further and act on immune cells and vagal gut innervations that communicate with the CNS to affect host behavior. In Chapter 5, I discuss the tools and multivariate investigative approaches employed in the studies discussed in this dissertation, and how multivariate approaches lend required dimensionality to studying a complex gut-brain signaling axis. Gut barrier dysfunction is a common theme observed in various disorders exhibiting altered anxiety behavior and gut dysbiosis of widely-varying microbial compositions. Understanding core functional features of gut dysbiosis will provide an important handle on ameliorating the gut environment in future attempts to treat CNS disorders.

INDEX WORDS: LPS, TLR4, Immune system, Gut-brain axis, Gut microbiota, Discriminant analysis

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DEDICATION

To my father and mother--Theodore Fields and Denise Fields. I am forever indebted to you for your belief in me and your never-ending support. Thank you.

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LIST OF ABBREVIATIONS

ANOVA, (univariate) analysis of variance

AVP, arginine vasopressin

ITS1/2, internal transcribed spacer regions 1 or 2

IL-1β, interleukin-1 beta

IL-6, interleukin-6

LPS, lipopolysaccharide

LPS-RS, lipopolysaccharide derived from *Rhodobacter spheroides*

MANOVA, multivariate analysis of variance

TLR2, toll-like receptor 2

TLR4, toll-like receptor 4

TNF-α, tumor necrosis factor-alpha

1 INTRODUCTION

1.1 Microbes and Mammals: A Symbiotic Relationship

From their earliest origins, eukaryotic cells have had a symbiotic relationship with microbes, which in multicellular organisms cover nearly every surface exposed to the environment, supporting critical aspects of host metabolism and physiology (Franco-Obregon and Gilbert, 2017). In humans, an estimated ~1:1 to 10:1 ratio of microbial cells for every human cell resides within the body, with the greatest reservoir being in the digestive tract (Sender et al., 2016). This microbial community is not only large by absolute number, but by complexity as well, and consists of myriad bacterial, fungal, viral, and protozoal species. Bacteria outnumber all other members, and of these, the *Bacteroidetes* and *Firmicutes* phyla predominate (Rosenbaum et al., 2015). However, broad generalizations about their impact on the host cannot easily be made as different species, and even strains within a specific phylum can differ markedly in physiology and metabolic output (Geva-Zatorsky et al., 2017). In addition, less abundant and even rare taxa may regulate overall community structure and function and play important roles in host physiology (Jousset et al., 2017; Enaud et al., 2018).

The largest reservoir of host-associated microbes resides within the gut lumen and are both impacted by the host's diet and play key roles in nutrient processing and host metabolism (Ley et al., 2008). Gut microbiota may also play a role in diet selection, guiding host preference for high-fat versus low-fat diets, or even inducing specific cravings (e.g. chocolate) (Rezzi et al., 2007; Alcock et al., 2014). The importance of gut microbiota to digestion is highlighted by the size difference in the cecum in germ-free versus conventionally colonized rodents (Savage and Dubos, 1968; Gustafsson and

Maunsbach, 1971). The cecum is a major nutrient processing center, particularly for soluble fibers, connecting the small and large intestines. The enlarged cecum observed in germ-free mice is a byproduct of the biomass of undigested fibers found in the host's diet (Iwai et al., 1973). However, the impact of gut microbiota on the host extends far beyond nutrient processing. Bacteria within the gut microbiota also produce and release important metabolic byproducts critical to maintaining homeostasis within the host enteric environment (Lin and Zhang, 2017). Short-chain fatty acids, such as butyrate, produced by certain lactic acid-releasing bacteria, is an important energy source for intestinal epithelial cells, and also downregulates intestinal inflammation (Ohira et al., 2017). Bacteria also release neurotransmitters, which likely play critical roles in communicating with the enteric nervous system, and perhaps also vagal gut innervations (Wall et al., 2014). Finally, of course, microbial components interact with the intestinal immune system, which is found in the largest reservoir of mammalian immune cells—namely the lamina propria (Shi et al., 2017). Alterations in the composition of the gut microbiota confer differential activation of the host intestinal immune system, which impacts the hosts varied organ systems (Pickard et al., 2017).

Over the past two decades, an explosion of research has begun to detail the robust relationship between gut microbiota and the CNS. Many of the foundational studies investigating the so-called gut-brain axis were made possible by the generation of germ-free rodents, which are devoid of microbes from birth. These animals demonstrate significant alterations in host physiology and behavior, suggesting that the microbiota communicates critical signals required for normal development (Mazmanian et al., 2005; Ley et al., 2006; Diaz Heijtz et al., 2011; Neufeld et al., 2011b). Additional

studies have manipulated content by either administering probiotics (microorganisms that promote host health, administered individually or as a cocktail), or antibiotics, or by direct bulk transfer of gut microbiota across model organisms. Such studies have revealed fundamental roles for the gut microbiota in modulating complex host behaviors, including social, stress-induced, and cognitive behaviors (Sudo et al., 2004; Diaz Heijtz et al., 2011; Neufeld et al., 2011b; Clarke et al., 2013). More recent studies also point to a role for microbiota in various neurological and psychiatric disorders, ranging from autism, Parkinson's disease, and substance use disorders (Hsiao et al., 2013; Kiraly et al., 2016; Sampson et al., 2016).

1.2 What Is Dysbiosis?

Dysbiosis of the gut microbiota, often referred to as "dysbiosis", and typically defined as a shift toward pathological, pro-inflammatory gut microbiota, has been linked to neuropsychiatric disorders, including autism (Finegold et al., 2012; Mayer et al., 2014b; Mayer et al., 2014a), ADHD (Petra et al., 2015), and psychological pathologies co-morbid with inflammatory bowel disease (IBD) (Bannaga and Selinger, 2015; Ray and Dittel, 2015). However, given the complex ecology of gut microbe-to-microbe and microbe-to-host interactions, precisely how changes in the gut microbiota influence behavior in these disorders remains largely unknown. To date, gut microbiota research involves largely correlational science, with any noted alterations in gut microbiota composition observed between healthy and diseased subjects described as "dysbiosis" (Olesen and Alm, 2016; Hooks and O'Malley, 2017). This is problematic given wide variations in microbial composition between healthy subjects. For example, the Human

Microbiome Project revealed that it is possible for two healthy individuals to harbor little to no overlap in gut microbial composition (Gilbert et al., 2018). A recent literature survey revealed that most authors that invoke the term dysbiosis only, at best, provide a vague definition. About half used the term to suggest an “imbalance” in the microbiota, one-fifth used the term to indicate an unspecified “change” (such as loss of diversity) and a quarter indicated specific taxa changes (such as an expansion of Proteobacteria and decrease in Firmicutes) (Hooks and O'Malley, 2017). In order for microbiota research to evolve into a more explanatory science, core mechanisms of action will need to be explored. Identifying common themes and functional consequences of what has been termed “dysbiotic” microbiota provides a first step for providing testable hypotheses and potential therapeutic targets for gut microbiota-associated disorders (Fields et al., 2018).

While there is currently no consensus on what constitutes dysbiosis, certain common themes tend to emerge. Core mechanisms underlying pathogenic actions of a dysbiotic microbiota may be breach of the gut barrier, exposure of the intestinal immune system to microbial components, and promotion of systemic inflammation. As will be described in Chapter 3, transfer of gut microbiota from donor mice exhibiting signs of intestinal inflammation induced by food emulsifier treatment into healthy mice induces the same breakdown of the gut barrier and intestinal inflammation in the recipient as that observed in the donor (Chassaing et al., 2015). There are other factors that may be involved in determining the health- or disease-promoting status of gut microbiota. For example, some bacterial species have consistently been identified to promote host health and have been deemed “probiotic”, including some *Lactobacillus* species. These

tend to downregulate chronic gut inflammation while promoting targeted immune responses to invasive pathogens (Dhama et al., 2017; Rocha-Ramirez et al., 2017). Other, more pro-inflammatory species such as certain Proteobacteria species may promote chronic gut inflammation (Litvak et al., 2017). Diversity within gut microbiota also limits the growth of these pathogenic species, and dysbiosis tends to be associated with lower levels of diversity of species composition (Morgan and Huttenhower, 2012; Weiss and Hennet, 2017). The studies discussed in this dissertation will focus on the effects of microbes that modulate gut barrier integrity, including lipopolysaccharide-shedding bacteria.

Gut inflammation, triggered by increased levels of LPS, may be a key component of gut-brain signaling in dysbiosis-associated disorders. Lipopolysaccharide (LPS) is a pathogenic component of gram-negative bacteria and is endogenous to the gut microbiota. Approximately half of the gut bacteria belong to the Bacteroidetes phylum, which is mostly gram-negative (Knight et al., 2017), and is a constitutive and dominant presence in the enteric environment. LPS is a likely candidate for initiating increases in intestinal permeability in conditions exhibiting gut dysbiosis. The intestinal epithelium is a single-cell layer separating the gut microbiota from the host. Directly underneath this epithelium is the lamina propria, housing the intestinal immune system. If the intestinal epithelium is breached, gut microbiota can initiate a robust intestinal immune response (Thaiss et al., 2016), which may initiate behavioral pathologies associated with gut dysbiosis. Therefore, antimicrobial defenses, such as the mucus layer, immunoglobulin A, and antimicrobial defense proteins, that are released within the gut lumen, sequester gut microbiota away from the intestinal epithelial wall and underlying lamina propria

(Takiishi et al., 2017). In addition, the intestinal epithelium and immune cells that reside within the lamina propria propagate an immune defense against microbes that breaches the mucus layer through activation of the innate immune system, which recognizes the presence of microbiota via a system of alarmin detectors, most notably the toll-like receptors (TLRs).

LPS is the primary agonist for TLR4 (Lu et al., 2008), and TLR4 signaling on intestinal epithelial cells (IECs) increases intestinal permeability (Li et al., 2013; Guo et al., 2015). Commensal gut microbiota express a mixture of both TLR4 agonistic and antagonistic LPS species (e.g. *R. Spheroides* produces an under-acetylated LPS species that, although it binds to TLR4, it does not trigger the downstream signaling pathways that initiate an immune response, and therefore functions basically as a TLR4 antagonist) (Hajjar et al., 2002; Martirosyan et al., 2013). However, a gut microbial shift toward a higher prevalence of pathogenic gram-negative bacteria, which express LPS structures that act as TLR4 agonists, may increase intestinal permeability.

Gut barrier dysfunction, induced by elevated gut levels of gram-negative bacteria and/or increased shedding of pathogenic LPS species, may increase anxiety behavior in the host through various pathways. Some of these pathways are illustrated in **Figure 1.1**, where microbial components activate gut immune cells that influence vagal and CNS circuits. Elevated gut levels of gram-negative bacteria have been reported for clinical populations that exhibit elevated anxiety, such as children with autism (Finegold et al., 2010) or celiac disease (Nadal et al., 2007). Furthermore, severity of gastrointestinal issues, likely linked to gut inflammation, has been reported to correlate with levels of anxiety behavior in these disorders (Mazurek et al., 2013; Hsiao, 2014;

Gracie et al., 2016; Reigada et al., 2016a). In addition, inflammatory bowel disease (IBD), which comprises of a set of diseases characterized by increased gut permeability (Michielan and D'Inca, 2015), is highly co-morbid with anxiety disorders (Bannaga and Selinger, 2015). This connection between intestinal permeability and anxiety behavior is not restricted to clinical populations. Exercise in healthy but untrained individuals increases markers of intestinal permeability (Worobetz and Gerrard, 1985; Peters et al., 1999; Jeukendrup et al., 2000; van Wijck et al., 2011) as well as produces short-term symptoms of anxiety (Rhodes et al., 2003; Campbell et al., 2009; Hopkins et al., 2012). These correlations between intestinal permeability and anxiety are also observed in various rodent disease models. The dextran sodium sulfate model of IBD and the maternal immune activation model of autism exhibit increased intestinal permeability (Hsiao et al., 2013; Xiao et al., 2016) as well as increased expression of anxiety behavior (Hsiao et al., 2013; Hassan et al., 2014). This correlation between increased intestinal permeability and anxiety behavior is also observed in other disease models, including chronic alcohol exposure (Chen et al., 2015; Pascual et al., 2015) and the high-fat diet model of obesity (Bruce-Keller et al., 2015). Interestingly, a probiotic treatment that reduces intestinal permeability also reduces anxiety behavior in the maternal immune activation model of autism (Hsiao et al., 2013).

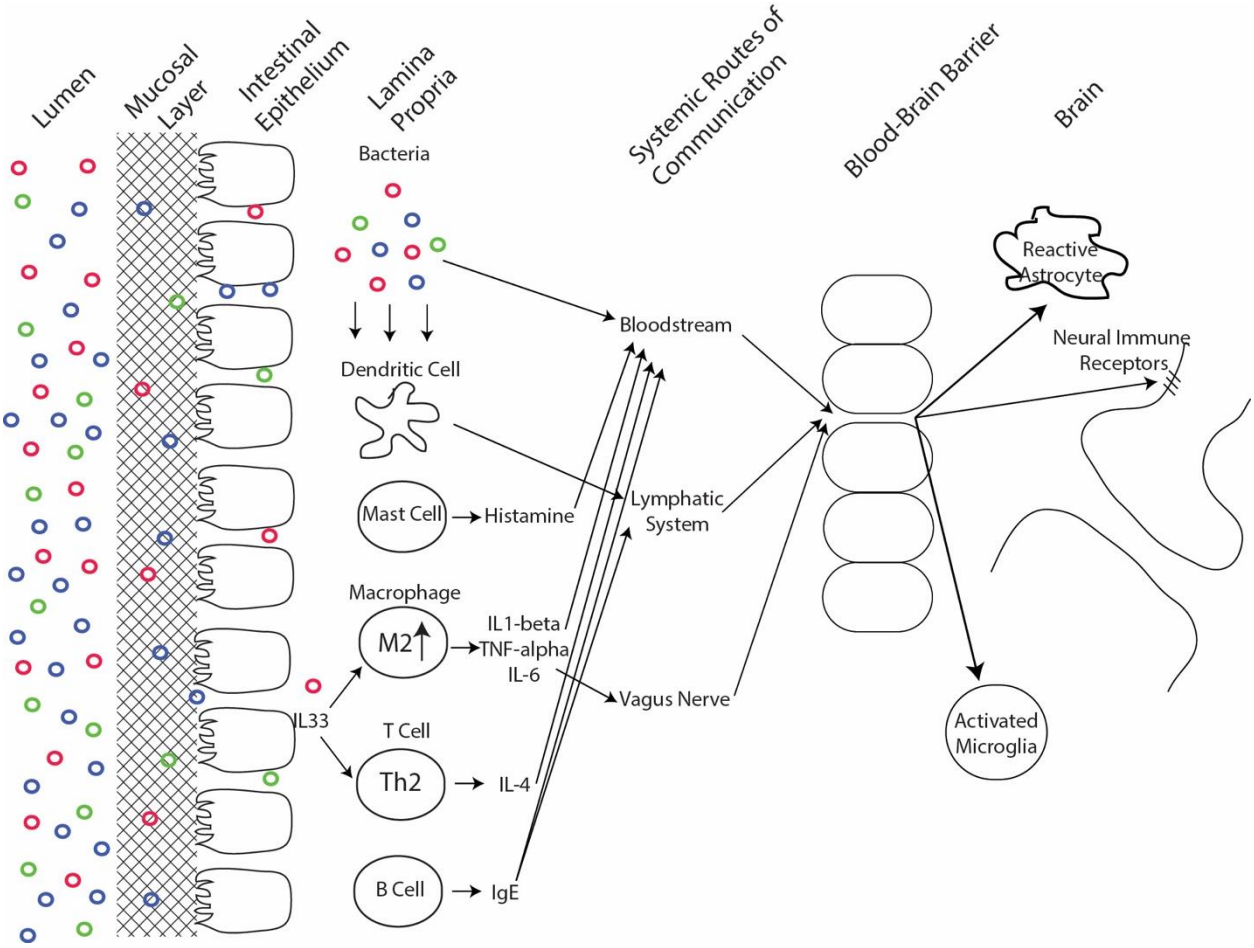

Figure 1.1 Model for how gut barrier dysfunction impacts the CNS.

Bacteria from the gut lumen (colored circles) penetrate the mucosal layer to interact with intestinal epithelial cells, downregulate intestinal epithelial tight junctions, and activate immune cells residing along the lamina propria, such as dendritic cells, mast cells, macrophages, T cells, and B cells. Dysbiosis, particularly the increased presence of enteropathogenic E Coli and other bacteria that penetrate the mucosal lining, triggers the production of IL-33 by intestinal epithelial cells which promotes a type 2 bias in immune cells in the lamina propria. Inflammatory mediators produced by these cells travel through the bloodstream and lymphatic system before reaching the blood-brain barrier. However, intestinal cytokines may also activate vagal nerves that, in turn,

trigger neuroinflammation through trans-synaptic signaling to immune *cells resident on the brain side of the blood-brain barrier. Inflammatory mediators that reach the blood-brain barrier can also penetrate and activate brain-resident astrocytes, microgliam, and immune receptors expressed on neurons.*

1.3 Summary of Chapters

The studies discussed in this dissertation will explore whether, and if so, how shifts in gut microbiota may contribute to anxiety-related behavioral pathologies. To approach this question, we needed to widen the tools of analysis typically employed in behavioral neuroscience studies to understand the complex implications of a multifaceted microbiome on multidimensional host behavior. The current state of microbiome analyses routinely uses a big-data approach to describe the composition and imputed functional implications of various gut microbial communities (Caporaso et al., 2010; Segata et al., 2011). There are two levels of analysis employed in this dissertation--one being a comparison of microbial population structure between sample groups and another a more microscopic view of the taxa that exhibit the most salient differences between sample groups. For the studies presented in this dissertation, we chose to employ some of the most robust tools to highlight taxa differences between groups, while maintaining a sensitivity to the fact that population differences may lie in more complex, nuanced differences between microbial populations that are not typified by individual taxa differences. This multi-layered approach to studying the microbiome also inspired our use of multidimensional (multivariate) analysis of complex syndromes of behavior. The typical approach to behavioral analysis involves looking at single

behavioral measures and imputing a larger emotional/motivational state change in the animals exhibiting this behavior. For example, the ratio of time spent with familiar vs novel conspecifics is the primary screen for social behavior defects designed for the three-chamber social interaction test (Nadler et al., 2004; Yang et al., 2011). However, social behavior has a number of individual components that make up the multidimensionality of these interactions. For example, play behavior is composed of locomotor and dyadic interactions, along with vocalizations, all of which combine into a recognizable pattern of behavior (Paul et al., 2016). Follow-up studies on genetic models of autism often fail to reproduce the core finding of an altered social behavior preference in the social interaction assay, such as findings for the Shank3 KO model of autism (Dhamne et al., 2017). However, these models may still reveal intricate patterns of behavioral alterations that are not apparent from examining any single measure of social behavioral. While there are a number of confounds that may explain inconsistent findings across studies, examining syndromic effects across multiple behavioral measures may yield consistent findings across cohorts. This mirrors the heterogeneity of psychiatric populations such as individuals with schizophrenia, wherein no single diagnostic test accurately diagnoses all schizophrenic individuals, who display a wide range of psychiatric traits (Jablensky, 2010). This has inspired multivariate approaches to diagnosis that identifies prodromal symptoms and that more reliably diagnoses schizophrenic patients with a combination of brain imaging and behavioral diagnostic tools (Davidson et al., 1999; Koch et al., 2015). In this dissertation, I will be applying multivariate analysis to study various effects of treatments altering gut microbiota on host behavior.

In Chapter 2, I performed an association study exploring variations in gut microbiota between genetic models where anxiety-related behaviors differ. Specifically, I explore the gut microbiota in Brattleboro rats, a model of social behavior disorders. Brattleboro rats are arginine-vasopressin knockout (*Avp-/-*) rats that exhibit lower levels of social and anxiety-related behavior relative to their wildtype (WT) counterparts. While these effects have been attributed to lack of vasopressin action on brain vasopressin receptors found in social and anxiety regulation centers in the brain, as well as on systemic physiological and immunological consequences of a lack of vasopressin systemically that may feedback on biobehavioral neuroimmune system, work in the past 20 years has demonstrated robust effects of gut microbiota on behavior. Vasopressin may influence gut microbiota composition which may independently influence host brain function and behavior. Therefore, we wanted to explore whether there are microbial differences across WT and KO Avp genotypes that may contribute to behavioral differences observed across these genotypes. For this, we used Quantitative Insights Into Microbial Ecology (QIIME) to study microbial population differences between homozygous and heterozygous Brattleboro, and wildtype (WT) Long-Evans, rats, comparing the evolutionary biomarker 16S rRNA as a marker of bacterial species. To identify the bacterial taxa that exhibit the most salient differences between genotype groups, we settled on the tool Linear Discriminant Analysis coupled with Effect Size (LEfSe). This revealed an interesting taxon, namely the *Lactobaccillus* genus, that may impact gut barrier function and impact how population differences across the three genotypes may influence host inflammation and CNS activity along the gut-brain axis.

While Chapter 2 is an example of a correlation study, in Chapter 3 we directly tested the role of gut dysbiosis by exploring the effects of treatments that affect gut microbial composition as well as gut barrier function on host behavior. Here, we added two different food emulsifiers to the diet, with similar but slightly diverging effects on gut physiology and behavior, i.e., carboxymethylcellulose (CMC) and polysorbate-80 (P80). Both emulsifiers reduced the thickness of the intestinal mucus lining and increased gut inflammation. We showed, however, that each has unique effects on gut microbiota composition and syndromic effects on behavior and physiology. This was a multi-investigator project, in which I contributed to the design and implementation of the data analysis by applying robust tools to identify the gut microbiota composition and behavioral syndromic effects, namely LefSe to identify specific taxa affected by emulsifier treatment and multiple discriminant analysis to identify syndromic effects across multiple behavioral measures. Teasing these CMC- and P80-specific effects apart contributed to a fuller understanding of the effects of food emulsifiers on the host. The results of this study suggest that alterations in gut microbiota can induce changes that affect the gut barrier can have effects on behavior, perhaps by increasing host immune interactions to specific “dysbiotic” alterations in gut microbiota.

In Chapter 4, I explored a potential mechanism of action for gut dysbiosis effects on anxiety behavior. Specifically, I studied the effects of the gut bacterial inflammagen LPS on behavior. LPS is commonly used to induce the sickness behavior response, which is the behavioral arm of systemic immune activation. Typical components of sickness behavioral include lethargy, increased anxiety, and social withdrawal, which are all adaptive behaviors hypothesized to limit social transmission of disease and to

conserve energy, thereby promoting recovery (Dantzer, 2001). In experimental settings, the typical mode of administration of LPS to induce this sickness response is via intraperitoneal or intravenous injections (Dantzer, 2009; Remus and Dantzer, 2016). This mode of administration increases systemic levels at least 150-fold over baseline serum endotoxin levels, which resembles LPS levels observed under septic shock rather than what is typically observed in conditions exhibiting gut dysbiosis (Hansen et al., 2000). Here, I sought to model "dysbiotic" changes in gut microbiota that exhibit a shift to more pathogenic species and thereby an increased shedding of LPS, and therefore I studied the effects of enterically-derived LPS on behavior. Here, we see that oral administration of LPS has a very specific effect on anxiety-like behavior without causing changes in locomotion typically observed in sickness behavior triggered by peripheral injections of LPS. Again, while we identified individual changes in behavior, we also identified syndromic effects of LPS on behavior, which highlighted the unique effects each of the emulsifiers had on sex differences in behavior in Chapter 3, and also the unique effects of LPS on mice lacking an important innate immune receptor that is the primary sensor of LPS (TLR4) in Chapter 4. As this study only focused on acute effects of this treatment on the host, this suggests that fluctuations in enteric endotoxin levels may actively modulate anxiety levels in the host. Breakdown of the gut barrier would increase enteric LPS exposure to portals of circulation residing in the lamina propria (lymph and blood vessels), to vagal afferents, and to lamina propria-resident immune cells that all interact with the CNS to induce changes in behavior.

Combined, these studies suggest that future research that focuses on core mechanisms of microbial communication along the gut-brain axis, in addition to

identifying specific changes in gut microbiota composition, may accelerate understanding of the impact of gut microbiota on the CNS. In Chapter 5, I will discuss how the current limitations in gut microbiota analysis can be overcome by exploring gut microbiota effects across various different conditions that may converge upon the same physiological and CNS/behavioral effects. Multivariate approaches to data analysis featured in the studies presented in this dissertation may help to overcome these limitations.

2 VASOPRESSIN DELETION IS ASSOCIATED WITH SEX-SPECIFIC SHIFTS IN THE GUT MICROBIOME

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2.1 Abstract

Brattleboro rats harbor a spontaneous deletion of the arginine-vasopressin (Avp) gene. In addition to diabetes insipidus, these rats exhibit low levels of anxiety and depressive behaviors. Recent work on the gut-brain axis has revealed that gut microbiota can influence anxiety behaviors. Therefore, we studied the effects of Avp gene deletion on gut microbiota. Since Avp gene expression is sexually different, we also examined how Avp deletion affects sex differences in gut microbiota. Males and females show modest but differentiated shifts in taxa abundance across 3 separate Avp deletion genotypes: wildtype (WT), heterozygous (Het) and AVP-deficient Brattleboro (KO) rats. For each sex, we found examples of taxa that have been shown to modulate anxiety behavior, in a manner that correlates with anxiety behavior observed in homozygous knockout Brattleboro rats. One prominent example is Lactobacillus, which has been reported to be anxiolytic: Lactobacillus was found to increase in abundance in inverse proportion to increasing gene dosage (most abundant in KO rats). This genotype effect of Lactobacillus abundance was not found when females were analyzed independently.

Therefore, Avp deletion appears to affect microbiota composition in a sexually differentiated manner.

2.2 Introduction

The neuropeptide arginine-vasopressin (AVP) is released from hypothalamic neurons into the bloodstream of mammals, where it regulates water balance and other autonomic functions (Knepper et al., 2015). However, AVP is also released within the brain where it influences social and anxiety-like behavior (Neumann and Landgraf, 2012) and modulate stress responses (Joels and Baram, 2009). The Brattleboro rat, which contains a base-pair deletion in the *Avp* gene that prevents functional AVP expression, is a model for studying the effects of AVP on behavior (Sokol and Zimmerman, 1982; Surget and Belzung, 2008). Many of the behavioral abnormalities observed in Brattleboro rats, such as decreases in anxiety-like behavior (Balazsfi et al., 2015) and abnormal social preference (Feifel et al., 2009), are assumed to result from the lack of direct activation of AVP-responsive behavioral circuits. However, systemic factors that may be influenced by AVP expression may also influence anxiety and social behaviors in this model. One such systemic factor may be the gut microbiome, which has recently been shown to influence both social and anxiety behaviors (Cryan and Dinan, 2012).

Gut microbial composition has been correlated with AVP expression. Depletion of gut micobiota with antibiotics decreases hypothalamic AVP expression (Desbonnet et al., 2015). This suggests that microbiota may influence AVP expression. However, there are multiple ways in which AVP expression could influence microbiota composition. For

example, AVP expression influences stress responses, systemic inflammation, and behaviors that could subsequently influence microbiota composition. It is plausible that AVP expression and gut microbial compositional changes that are influenced by AVP expression could reinforce each other in a positive feedback loop.

This study seeks to establish whether there are compositional differences in gut microbiota between AVP knockout rats and wildtype (WT) rats. In addition, as AVP expression is also sexually dimorphic, with male rodents expressing more than female rodents in centrally-releasing projections as well as in neurosecretory cells (de Vries, 2008; Taylor et al., 2012), we sought to observe the effects of AVP deletion on sex differences in gut microbiota. Therefore, the objectives of this study were: i) to compare microbiota composition across AVP deletion genotypes (homozygous knockout, heterozygous, and wildtype Long Evans rats) and ii) to identify changes in sex differences of the microbiota upon haploid or diploid deletion of the AVP gene.

2.3 Materials and Methods

2.3.1 Experimental design and fecal collection

Brattleboro rats carrying a homozygous (KO) or heterozygous (Het) deletion of the AVP gene against a Long-Evans background, along with wildtype (WT) Long-Evans rats, were bred from Het breeding pairs. Offspring from eleven litters resulting from eleven separate breeding pairs, all born within a five-day span, were used in this study, yielding a total of 42 subjects. Upon weaning, all offspring were genotyped and pair-housed with the same genotype and sex. Prior to this study, at around 4 weeks of age, the rats were used in a play testing study (Paul et al., 2016). These rats endured no

further manipulations prior to the study. All of the animals were pair-housed with the same genotype and sex at the beginning of the study. We did not want to disturb this pairing in order to avoid the additional confound of introducing socially novel cage mates, which may independently affect microbial composition. The rats were housed in two separate subspaces of a housing room with generally regular exposure to the same set of researchers and environmental cues. The rats were housed in cages with bedding, fed non-autoclaved rat chow, given one nylabone and plastic shelter per cage for enrichment, and kept on a 12L:12D light cycle.

At twelve weeks of age, subjects from each cage were chosen at random and were single-housed into clean cages for 16-24 h. Three to four fecal pellets per cage were then collected with ethanol-cleaned forceps and promptly stored at -80°C. From each litter, no more than one rat per experimental group was used, with seven of the eleven litters producing animals from all three genotypes used in the study. With the exception of four animals, cage mates were not used (i.e. only one rat per pair housed cage was used in the study).

2.3.2 DNA extraction and 16s rRNA sequencing

Fecal microbial 16s rRNA was sequenced according to the protocol outlined in (Chassaing et al., 2015). Briefly, total bacterial DNA was isolated from feces using QIAamp DNA Stool Mini Kit (Qiagen) according to manufacturer's instruction and was stored at -80°C before further analysis. The 16S rRNA genes, region V4, were PCR amplified using the 515F/806R primer set (see ref. Chassaing et al., 2015 for full sequence). PCR reactions consisted of Hot Master PCR mix (Five Prime), 0.2 uM of each primer, 10-100 ng template, and reaction conditions were 3 min at 95 °C, followed

by 30 cycles of 45 s at 95 °C, 60 s at 50 °C and 90 s at 72 °C on a Biorad thermocycler. PCR products were purified with Ampure magnetic purification beads (Agencourt). Sequencing was performed on an Illumina MiSeq sequencer (paired-end reads, 2 x 250 base pairs) at Cornell University, Ithaca.

2.3.3 Bioinformatics and statistical analysis

The sequences were demultiplexed, quality filtered using the Qualitative Insights Into Microbial Ecology (QIIME, version 1.8.0) software package, and forward and reverse reads were joined using the fastq-join method (http://code.google.com/p/ea-utils) (Caporaso et al., 2010). Sequences were assigned to OTUs (Operational Taxonomic Units, a proxy for species classification, grouping closely related individuals) using the UCLUST algorithm with a 97% threshold of pairwise identity, and classified taxonomically using the Greengenes reference database (http://greengenes.lbl.gov) using uclust method with the suppression of new clusters (closed reference OTU picking strategy). FastTree was used to generate a phylogenetic tree and to compute unweighted UniFrac distances per sample (http://microbesonline.org/fasttree/). OTUs that were assigned to only one read for a sample were excluded from analysis. Principal coordinate analysis (PCoA) plots, constructed with weighted UniFrac distances, were used to assess the variation between experimental groups (beta diversity) and jackknifed beta diversity was used to estimate the uncertainty in PCoA plots. Metagenomic data prediction of the functional profiles of fecal microbial composition was generated using PICRUSt (Langille et al., 2013).

Measures of alpha diversity were compared across groups using the Mann-Whitney U test of significance. Significant tests of beta diversity difference between

sample groups were obtained using PERMANOVA in QIIME. The program Linear discriminant analysis (LDA) coupled with effect size (LEfSe) was used to identify significantly differentiated bacterial taxa (Segata et al., 2011). LEfSe was also used to analyze differential abundance in gene pathways between microbial samples predicted by PICRUSt. Bootstrap Kruskal-Wallis-test was used to identify taxa or gene pathways with significantly differentiated abundance, with the LDA score computed with a bootstrapping algorithm repeated over 30 cycles, each sampling two-thirds of the data with replacement. Unless otherwise stated, one-against-all multiclass analysis was utilized, and posthoc Wilcoxon pairwise comparisons among subclasses were only performed among identically named subclasses: in cross-genotype analyses, males were only compared with males and females only compared with females; in cross-sex analyses, subjects of the same genotype were compared to each other. The one-against-all algorithm detects whether at least one of the classes is significantly different from the other compared classes. However, the all-against-all algorithm detects whether all of the classes are significantly different from each other. The threshold on the logarithmic LDA (linear discriminant analysis) score for discriminative features was set to 2.0 (indicating significant differential abundance between classes), and the alpha values for the factorial Kruskal-Wallis test among classes and the pairwise Wilcoxon test between subclasses were both set to 0.05.

2.4 Results

2.4.1 Metadata

Long Evans rats with heterozygous expression of a functional and nonfunctional copy of the arginine-vasopressin gene (*Avp*) were bred to produce subjects expressing wildtype (WT), heterozygous (Het) and homozygous knockout (KO) variants of the *Avp* gene deletion. A total of 42 fecal samples (6 WT male, 8 WT female, 6 Het male, 7 Het female, 8 KO male, and 7 KO female) were collected with one sample per subject, from which DNA was amplified and sent for sequencing. After OTU picking and checking for chimeric transcripts, a total of 1,322,857 reads were assigned to 4,189 OTUs. Each sample has an average of 31,497 reads.

2.4.2 Differences in bacterial communities between Avp deletion genotypes

Gut microbial richness was not statistically different across the three *Avp* deletion genotypes. Between genotypes, we found no difference in any of the three measures of alpha diversity, which measures community richness (Shannon's diversity index, observed species and Chao1), when all data points were combined nor when genotypes were analyzed for each sex separately (data not shown).

The relationships between global microbiota composition were examined using Principal Coordinate Analysis (PCoA) based on weighted UniFrac distances. With males and females combined, weighted UniFrac-based cluster analysis reveals modest but differentiated microbiota compositions for each genotype **(Figure 2.1A).** The observed clustering of each group was confirmed by PERMANOVA (p=.024). When males and females were analyzed independently, clustering by genotype was observed

(Figures 2.1B and 2.1C). With weighted UniFrac distances, there are trends in differentiation by genotype for both males and females (p=0.051 and 0.071, respectively). These data suggest that the microbial community structures found in the guts of WT, Het, and KO Brattleboro rats are differentiated across a limited number of taxa.

We used LEfSe to identify specific bacterial taxa that are significantly differentiated between groups. All features identified by LEfSe exceed an LDA score of 2.0, which indicates significant differences between groups. **Figure 2.2** shows bacterial taxa differentially represented between genotypes, using the one-against-all algorithm which identifies taxa that are only differentiated in one genotype relative to the other two genotypes. When both sexes are analyzed together, *Lactobacillus spp.* are most abundant in KO rats and *Blautia producta* is most abundant in Het rats (**Figure 2.2A**). When male samples were analyzed separately by genotype, the same taxa were differentiated, with the addition of *Desulfovibrio c21_c20* being more abundant in KO rats (**Figure 2.2B**). When female samples are analyzed independently, *Lachnospira spp.* were most abundant in Het rats while *Holdemania spp.* were most abundant in WT rats (**Figure 2.2C**). Using the all-against-all algorithm within LEfSe, which identifies features that are significantly differentiated among all pairwise comparisons, we found zero significantly differentiated taxa between genotypes when both sexes are combined**.** However, when the sexes were analyzed separately, significantly differentiated taxa between genotypes are identical to those identified with the one-against-all algorithm. For example, *Lactobacillus* is significantly differentiated between all three genotypes among male rats but is not significantly differentiated among female rats **(Figure 2.3)**.

The all-against-all LEfSe algorithm indicates that the relative abundance of *Lactobacillus* is differentiated across all three genotypes for males (LDA score = 4.6), and the average abundance for each class increases with haploid and diploid deletion of the *Avp* gene. In keeping with differentiated clustering of Het animals identified via PCoA plots, this LDA analysis suggests that Het rats harbor a microbiota that is differentiated from that found in WT and KO rats, particularly for these bacterial taxa.

Using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), we explored the predicted functional consequences of these compositionally differentiated microbiota for males and females separately. OTU table was normalized by 16S rRNA copy number and gene pathways were predicted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. This generated the pathway abundance table that was analyzed by LEfSe. Males show more differentiation in pathways between genotypes. In males, 6 pathways were most abundant in KO rats (e.g. “Ribosome biogenesis in eukaryotes”, aminoacyl_tRNA_biosynthesis”, “Xylene degradation”, etc.), 9 pathways most abundant in Het rats (e.g. “Genetic information processing”, “Translation”, and “Ribosome”, etc.) and 8 pathways most abundant in WT rats (e.g. “Other glycan degradation”, “Sphingolipid metabolism”, “Biosynthesis of other secondary metabolites”, etc.) (**Figure 2.4A**). Between female rats, “Glycolysis and Gluconeogenesis” is most abundant in KO rats, “Amino acid metabolism”, “Valine Leucine and isoleucine biosynthesis”, “Pantothenate and CoA biosynthesis” are most abundant in Het rats, and “Phenylalanine, tyrosine and tryptophan biosynthesis”, “Arginine and proline metabolism”, “C5 branched dibasic acid metabolism” are most abundant in WT rats (**Figure 2.4B**).

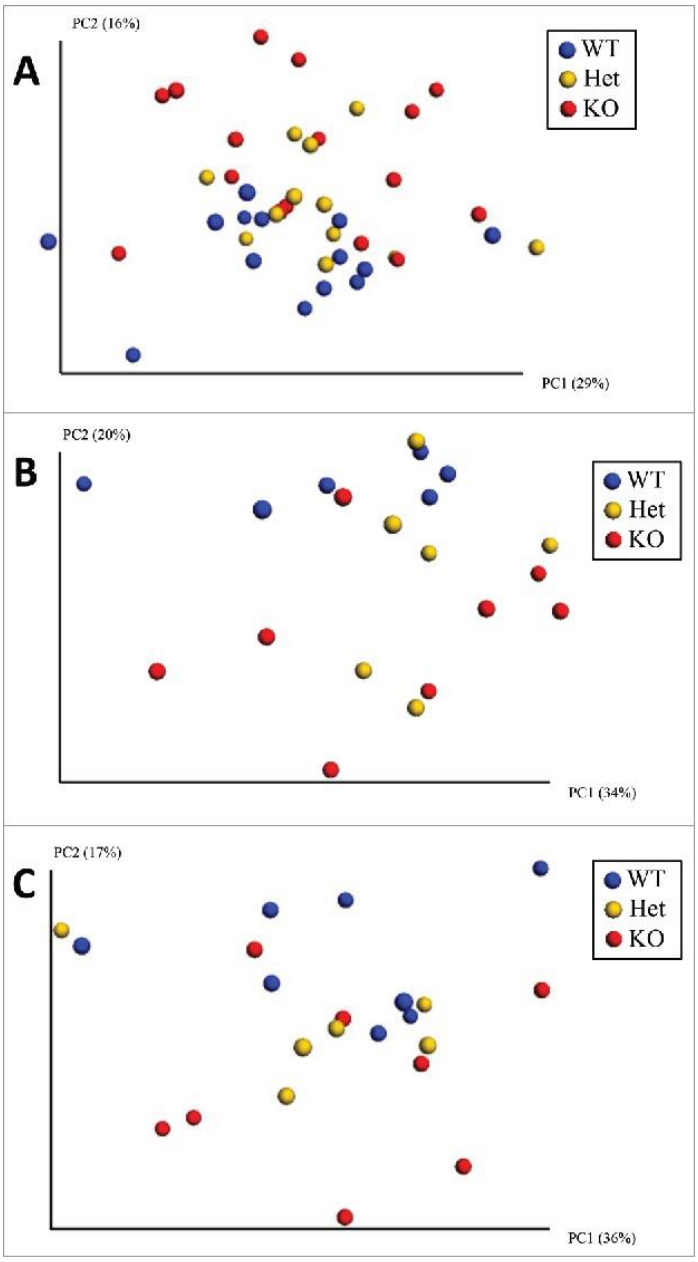

Figure 2.1 Clustering of gut microbial populations from Brattleboro rats by genotype.

Covariation of community structure using weighted UniFrac distances demonstrates limited clustering of samples by genotype when (A) both sexes are analyzed together [KO are clustered in upper right, WT are clustered in lower left, while Het are found in

the middle; PERMANOVA, p< = 0.05] and when (B) males [PERMANOVA, p = 0.051] and (C) females [PERMANOVA, p = 0.071] are analyzed separately.

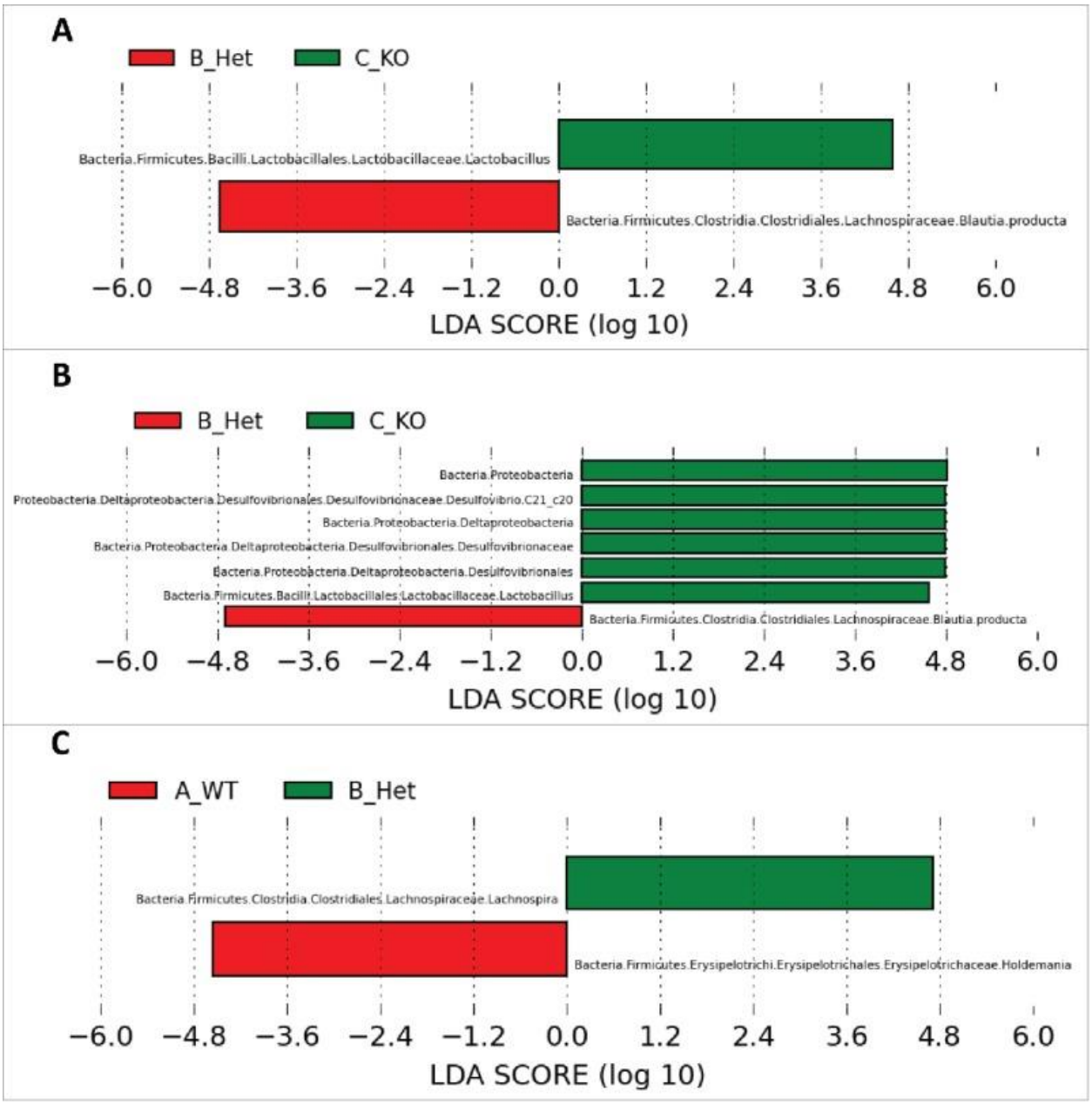

Figure 2.2 Bacterial taxa significantly differentiated between genotypes identified by Linear Discriminant Analysis (LDA) coupled with Effect Size (LEfSe).

(A) shows differentiated taxa between WT, Het, and KO rats when males and females are combined. (B) and (C) show differences between genotypes for males and females, respectively. All LDA scores exceed 2.0, which is the threshold for significantly differentiated features.

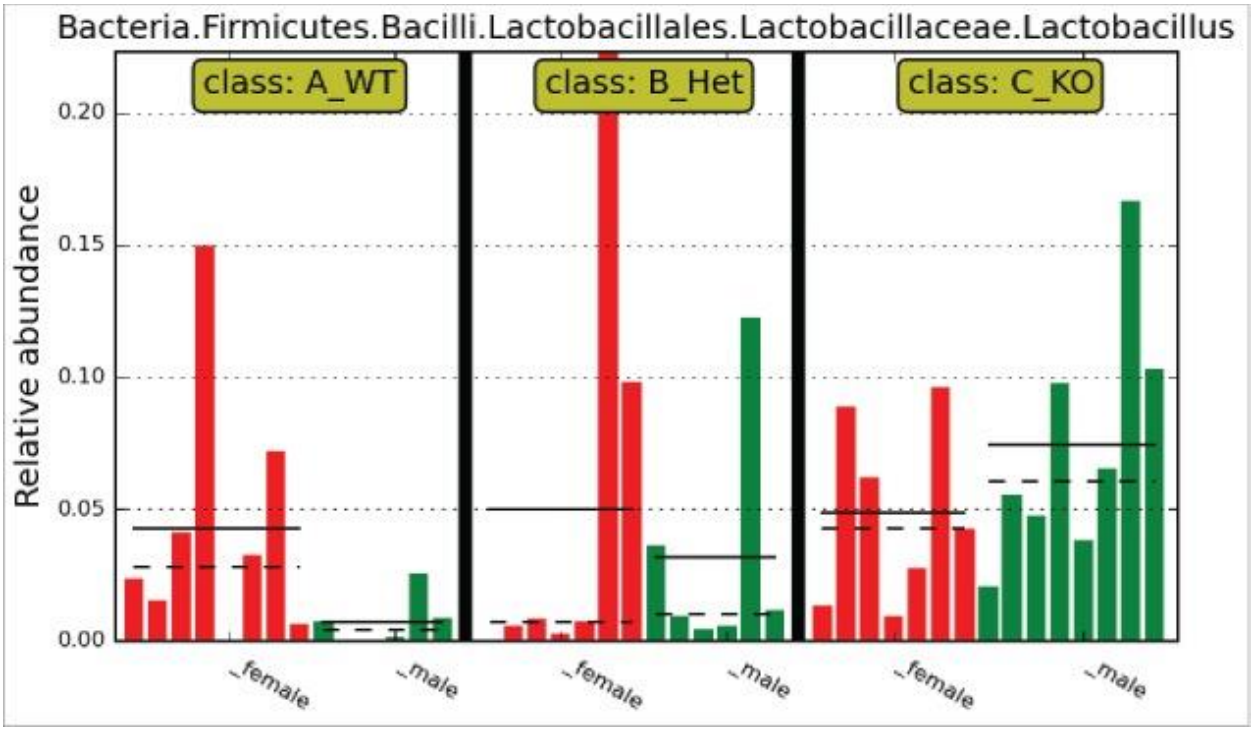

Figure 2.3 Relative abundance of Lactobacillus taxon between genotypes.

All-against-all algorithm of Linear Discriminant Analysis (LDA) coupled with Effect Size (LEfSe) identifies this taxon as significantly differentiated between all genotypes [WT, Het, and KO] for male rats. (LDA score = 4.6, which exceeds the score threshold of 2.0, indicating statistical significance). Neither the all-against-all or one-against-all algorithms detect Lactobacillus as a significantly differentiated taxon between genotypes for female rats.

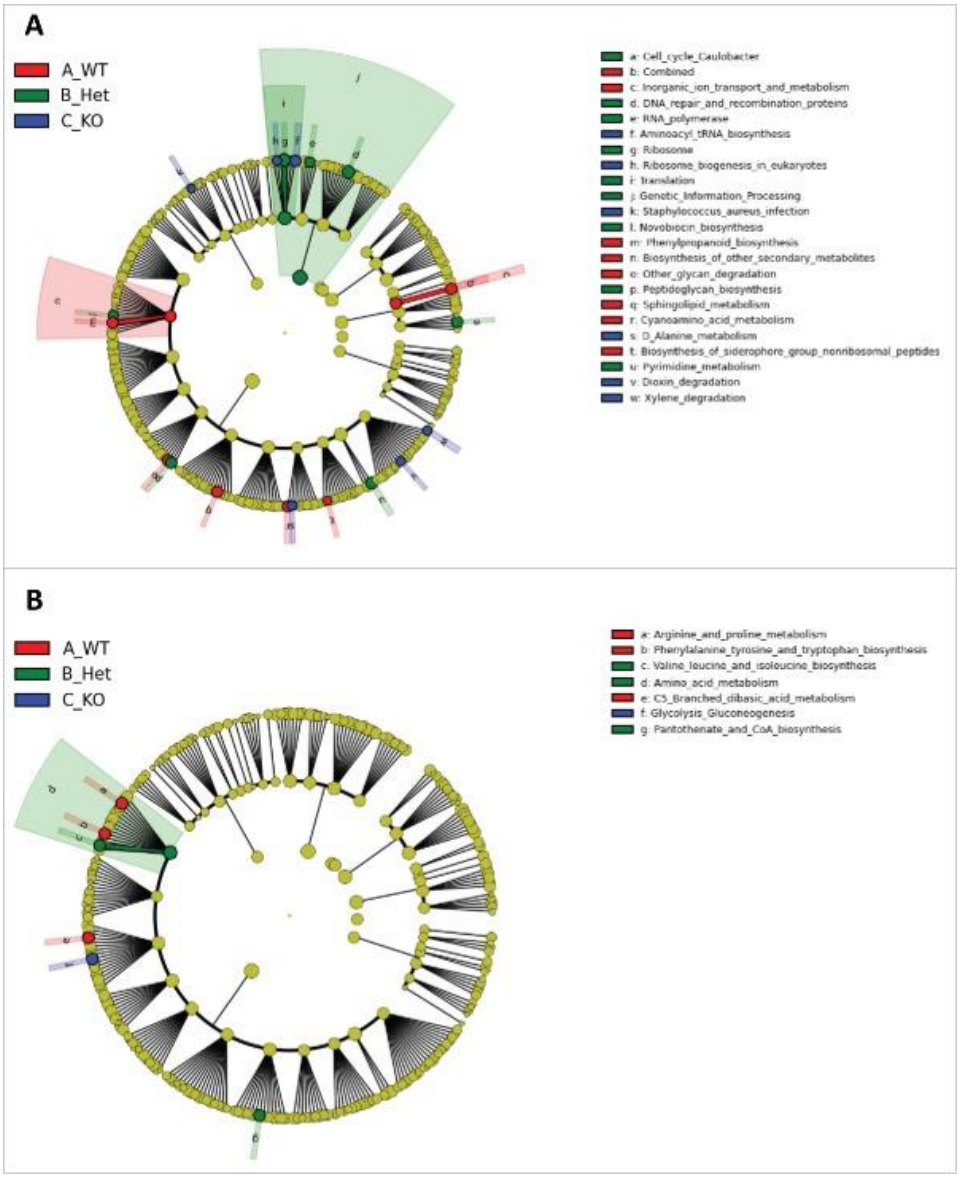

Figure 2.4 Cladogram of gene pathways significantly differentiated between genotypes identified by Linear Discriminant Analysis (LDA) coupled with Effect Size (LEfSe).

The innermost ring represents KEGG Level 1 pathways, the middle ring represents KEGG Level 2 pathways, and the outermost ring represents KEGG Level 3 pathways. (A) and (B) show predicted functional differences between genotypes [WT, Het, and

KO] for males and females, respectively. All highlighted pathways have LDA scores that exceed 2.0, which is the threshold for significantly differentiated features.

2.4.3 Differences in bacterial communities between sexes

When considering overall community composition via PCoA analysis, we observe no sex differences in gut microbiota. When comparing alpha diversities between sexes with all of the genotypes combined, or between sexes for each separate genotype, no significant differences in species diversity were observed. No sex differences in overall community composition were identified via PCoA analysis of all of the samples combined, or for any of the three separate genotypes (data not shown).

At the level of individual taxa (as analyzed via LEfSe), we were able to identify sex differences across all three genotypes. Between WT males and females, *Dorea spp.* and *Ruminococcus spp.* are more abundant in female rats (**Figure 2.5A**). This sex difference in community composition is altered in Het and KO rats. Among Het rats, *Odoribacter spp.*, *Lactobacillaceae spp.* and *Dehalobacterium spp.* are more abundant in females, whereas *Granulicatella spp.* and *Blautia producta* are more abundant in males (**Figure 2.5B**). Among KO rats, *Lactobacillaceae spp.*, *Dehalobacterium spp.*, and *Eubacterium dolichum* are more abundant in females (**Figure 2.5C**).

The metabolic potentials between sexes for each genotype were explored using PICRUSt-generated BIOM tables analyzed via LEfSe. We were only able to identify one gene pathway category that is sexually differentiated across each of the three distinct genotypes. In WT rats, “Secretion Systems” predominate in females (**Figure 2.6A**), whereas in Het rats, “RNA polymerase” pathways are most abundant in males (**Figure**

2.6B). These pathways are not sexually differentiated in KO rats, where an unclassified group of pathways is most abundant in females (**Figure 2.6C**).

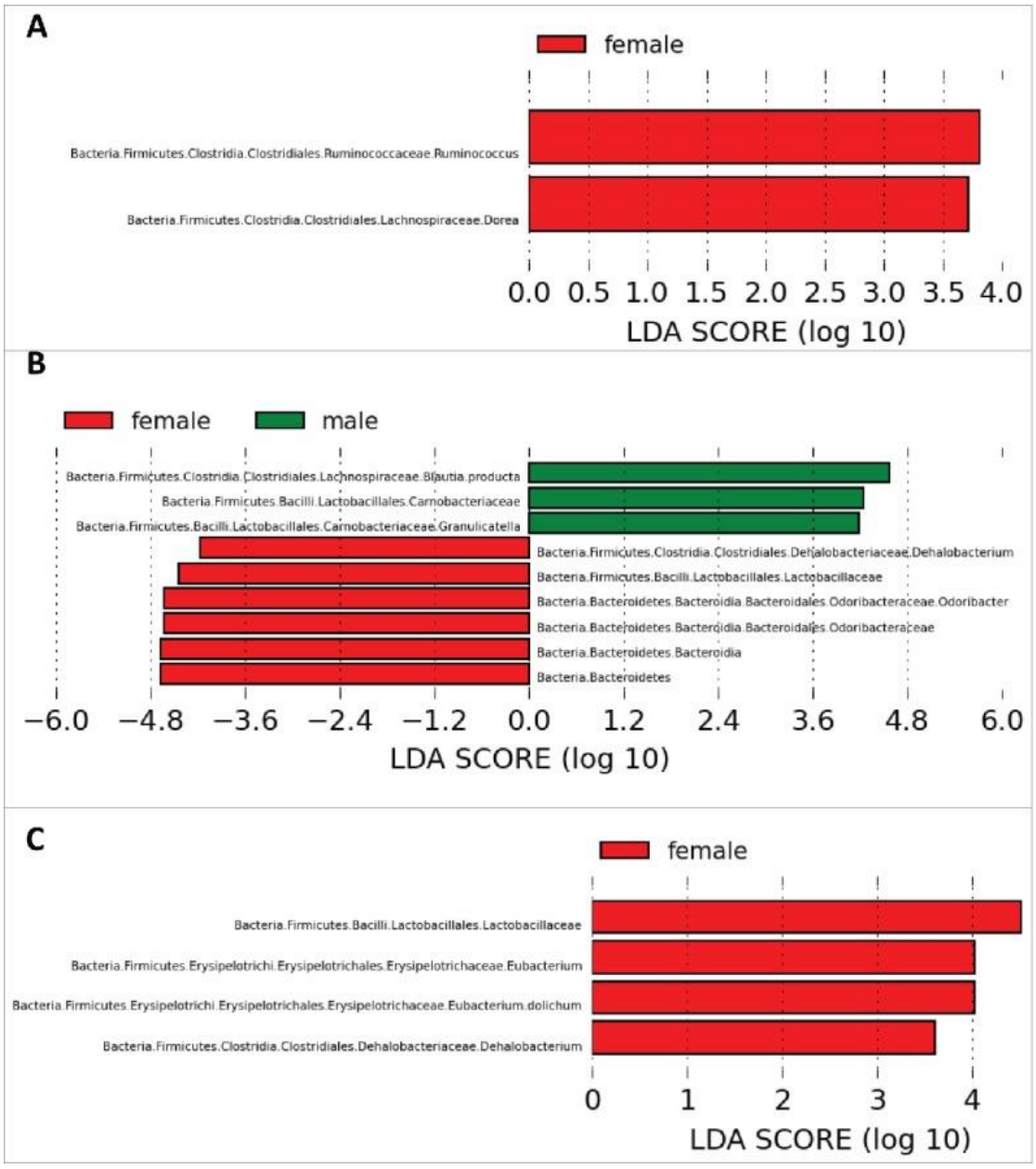

Figure 2.5 Bacterial taxa significantly differentiated between sexes identified by Linear Discriminant Analysis (LDA) coupled with Effect Size (LEfSe).

Differentiated taxa between males and females of the (A) WT, (B) Het and (C) KO genotypes are shown. All LDA scores exceed 2.0, which is the threshold for significantly differentiated features.

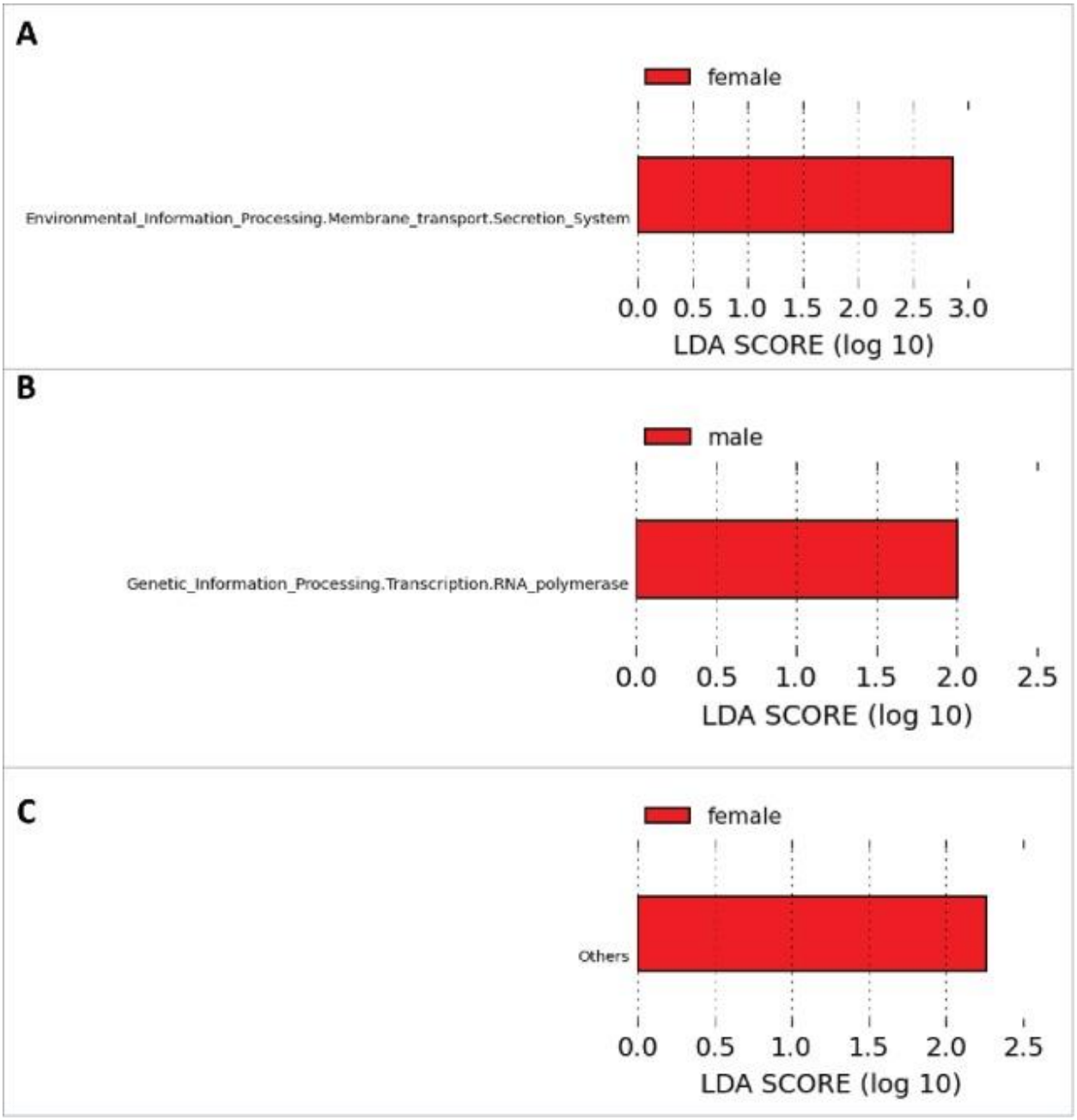

Figure 2.6 Gene pathways significantly differentiated between sexes identified by Linear Discriminant Analysis (LDA) coupled with Effect Size (LEfSe).

Differentiated taxa between males and females of the (A) WT, (B) Het and (C) KO genotypes are shown. All highlighted pathways have LDA scores that exceed 2.0, which is the threshold for significantly differentiated features.

2.5 Discussion

This study is the first to identify differences in gut microbiota between arginine-vasopressin (AVP) deletion genotypes: namely homozygous (KO), heterozygous (Het) and wildtype (WT) Brattleboro rats. We found differences in microbiota across all three genotypes, suggesting that *Avp* is haploinsufficient to restore microbiota observed in WT rats. Interestingly, we also found that sex differences in gut microbiota were affected by *Avp* genotype.

Breeding genetic knockout and WT colonies in isolation may result in compositional differences in gut microbiota that are not truly reflective of genotype effects on microbiota composition (Ubeda et al., 2012). We avoided this confound by generating all genotypes used in this study from heterozygous breeding pairs. In order to ensure that fecal samples were only collected from the subject animal and not from a cagemate, subject animals were single-housed for 18-24 hours prior to sample collection. Single-housing can affect stress reactivity (Das et al., 2015) and chronic stress exposure could potentially alter microbiota composition (Stilling et al., 2014). We reasoned that 24 hour separation would not significantly alter microbiota composition, as large scale differentiation of microbiota composition require several days in other models (Mason et al., 2012; Stilling et al., 2014; Chassaing et al., 2015). All animals were subjected to the same single-housing protocol.

Our data suggest that haploid or diploid expression of the *Avp* gene differentially affects the abundance of specific bacterial taxa, and that it does so in a sex-specific manner. Microbial differences were detected with QIIME via PCoA analysis to determine whether large scale microbial population differences exist between groups

(Navas-Molina et al., 2013) and with LEfSe, a very conservative biomarker discovery tool which detects the most robust differences between groups (Segata et al., 2011; Paulson et al., 2013), which are likely to be the most influential to explaining differences in host physiology and behavior. Both PCoA analysis and LEfSe indicate that each genotype is significantly differentiated from the other. Also, females exhibit a separate set of differentially abundant taxa between genotypes relative to those found in males. Unique findings of sex-specific compositional differences between genotypes are supported by analysis of microbiota composition by sex. The sexually differentiated taxa found in WT rats are not observed in Het and KO rats, and vice versa. PICRUSt analysis, which demonstrates differences in the functional capacity of gut microbiota, also suggests a unique microbiota for Het rats and highlights the effects of subject sex on genotype differences in microbiota composition. It is important to note that while PICRUSt has demonstrated a high level of predictive validity in mammalian microbial samples, PICRUSt analyzes data from a “closed-reference” subset of the original community composition BIOM table, and the accuracy of PICRUSt predictions still lie between 60-90% for mammals (Langille et al., 2013).

AVP KO rats show less anxiety behavior than WT rats (Balazsfi et al., 2015). Our data suggest this may, in part, be driven by the higher abundance of *Lactobacillus spp.* found in the gut microbiota of KO rats relative to WT rats. Oral administration of *Lactobacillus spp.* decreases anxiety behavior in mice and rats (Bravo et al., 2011; Mackos et al., 2013; Ohland et al., 2013; Luo et al., 2014; Liang et al., 2015; Liu et al., 2015; Wang et al., 2015b). Of note, male Het rats show levels of *Lactobacillus spp.* intermediate to those found in male WT and male KO rats. As *Avp* is haploinsufficient in

restoring normal working memory (Aarde and Jentsch, 2006) and in selective parameters of developmental behavior (Paul et al., 2016), a thorough investigation of differences in other behaviors such as anxiety behavior between Het and WT rats is warranted. The anxiety modulating properties of *Desulfovibrio spp.* and *Blautia producta*, most abundant in KO and Het rats respectively, have not been investigated in conventional WT rats. However, a gnotobiotic mouse model solely colonized with a *Blautia sp.* demonstrates decreases in marble burying behavior and moderate decreases in time spent in the periphery of the open field test relative to germ-free mice, suggesting decreases in repetitive and anxiety-like behaviors in these mice (Nishino et al., 2013). This is particularly notable, as germ-free mice show decreased anxiety-like behavior with respect to conventionally colonized mice (Diaz Heijtz et al., 2011; Neufeld et al., 2011b).

Some differentiated taxa that have been associated with weakened immune systems or with inflammatory states are more abundant in KO or WT rats, respectively. AVP is important for shaping immune responses, and rats with a homozygous *Avp* deletion harbor a hyporesponsive immune system, showing deficits in macrophage activation, IgG antibody response, a smaller spleen and premature involution of the thymus (Khegai et al., 2003). *Desulfovibrio c21_c20*, found most abundantly in male KO rats relative to male WT rats, is a bacterial species of the Proteobacteria phylum, which has been found to be highly abundant in mice with a disruption in their innate immune system (namely, toll-like receptor 5 which recognizes flagellated bacteria) (Carvalho et al., 2012). Between female rats, *Lachnospira spp.* are most abundant in Het rats and *Holdemania spp.* are most abudnant in WT rats. *Holdemania* is a genus of the

Erysipelotrichales order; Erysipelotrichales bloom in response to a high-fat diet (Magnusson et al., 2015), which promotes intestinal inflammation. Children with asthma have a lower abundance of *Lachnospira spp*. in their gut microbiota, and germ-free mice colonized with a *Lachnospira* species show decreases in airway inflammation (Arrieta et al., 2015). Given the two-way relationship between microbiota and the immune system (Lei et al., 2015; Tomkovich and Jobin, 2015), it is possible that *Lachnospira spp*. suppress inflammation in a commensally beneficial manner that promotes further replication of *Lachnospira spp.* in female KO rats.

One mechanism by which *Avp* deletion may alter gut microbiota is via regulation of water consumption. Drinking water conditions, such as the pH of consumed water, can alter gut microbiota (Sofi et al., 2014; Wolf et al., 2014). As AVP is important for water retention, Brattleboro rats display signs of *diabetes insipidus*, i.e. increased water intake and urine output. However, restoring systemic AVP levels via osmotic minipumps, which corrects water balance and diabetes symptoms, does not normalize anxiety and depressive behaviors in Brattleboro rats (Balazsfi et al., 2015). In addition, the heterozygous Brattleboro condition is sufficient to correct for outward signs of *diabetes insipidus* (Laycock, 1977; Opava-Stitzer et al., 1982), but the heterozygous condition is still unable to correct working memory deficits that are observed in homozygous knockout Brattleboro rats (Aarde and Jentsch, 2006). This suggests diabetes symptoms such as water consumption are not the sole driver of behavioral differences between Brattleboro and WT rats.

There are other potential mechanistic links between AVP expression and microbiota composition. It is possible that maternal behaviors such as pup licking-

grooming affect microbiota composition. KO Brattleboro dams have been demonstrated to exhibit maternal neglect, spending less time licking and grooming their pups than Het dams (Fodor et al., 2012). However, all subjects in this study were raised by Het dams. Nevertheless, KO pups may elicit differing levels of maternal licking-grooming behavior than Het and WT rats. KO rats exhibit differing levels of ultrasonic calls relative to WT and Het rats (Paul et al., 2016) and pup ultrasonic calls may be associated with rates of licking and grooming (Brouette-Lahlou et al., 1992), which may potentially affect adult gut microbiota composition.

Moving from behavior to cellular biology, AVP may directly affect microbiota composition via receptors present on bacteria that may be structurally similar to host neurotransmitter/neuropeptide receptors (Corringer et al., 2012). Indeed, many neurotransmitters are suggested to derive from bacterial origins through lateral gene transfer into the metazoan lineage (Iyer et al., 2004). An *in vitro* study found that AVP was stable in a colonic environment devoid of fecal microbiota (Wang et al., 2015a). Therefore, AVP may be metabolized by the microbiota in a manner that influences their growth, cell death, or functional output, and may subsequently affect the host.

AVP release, potentially both centrally and systemically, modulates the activity of immune cells (Shibasaki et al., 1998; Hu et al., 2003) and AVP-producing nuclei are responsive to inflammatory stimuli (Nava et al., 2000). Many immune cells also express AVP receptors (Russell and Walley, 2010). Similar to AVP, gut microbiota both regulate, and are shaped by, the immune system (Lei et al., 2015; Tomkovich and Jobin, 2015). Therefore, there may be a bidirectional link between gut microbiota and AVP expression mediated by the immune system. Future studies could investigate differences in

behavioral and cytokine profiles in germ-free rats administered microbiota from WT vs KO Brattleboro rats.

In summary, we characterized the gut microbiota of wildtype (WT) Long Evans rats and Long-Evans rats carrying haploid (heterozygous, Het), or diploid (knockout, KO) deletions of the *Avp* gene, and found a limited but potentially influential subset of significantly differentiated taxa that correspond with the immune status and anxiety behavior differences observed between WT and KO rats. Rats heterozygous for the *Avp* gene harbor a unique microbiota, that appears to be intermediate to that found in the guts of WT and KO rats. *Avp* gene deletion appears to affect the community composition of the gut microbiota of males and females in a sexually differentiated manner. Future studies should more fully explore the behavioral phenotype of Het rats relative to WT rats, and how sex differences in behavior are altered by *Avp* gene deletion.

3 DIETARY EMULSIFIERS CONSUMPTION ALTERS ANXIETY-LIKE AND SOCIAL-RELATED BEHAVIORS IN MICE IN A SEX-DEPENDENT MANNER

Mary K. Holder, Nicole V. Peters, Jack Whylings, Christopher T. Fields, Andrew T. Gewirtz, Benoit Chassaing, and Geert J. de Vries

Slightly modified from Holder, Mary K., et al. “Dietary emulsifiers consumption alters anxiety-like and social-related behaviors in mice in a sex-dependent manner” Scientific Reports x.x (2018): x-x.

3.1 Abstract

Dietary emulsifiers carboxylmethylcellulose (CMC) and polysorbate 80 (P80) alter the composition of the intestinal microbiota and induce chronic low-grade inflammation, ultimately leading to metabolic dysregulations in mice. As both gut microbiota and intestinal health can influence social and anxiety-like behaviors, we investigated whether emulsifier consumption would detrimentally influence behavior. We confirmed that emulsifier exposure induced chronic intestinal inflammation, increased adiposity, and altered gut microbiota composition in both male and female mice, although the specific microbial taxa altered following emulsifier consumption occurred in a sex-dependent manner. Importantly, emulsifier treatment altered anxiety-like behaviors in males and reduced social behavior in females. It also changed expression of neuropeptides implicated in the modulation of feeding as well as social and anxiety-related behaviors. Multivariate analyses revealed that CMC and P80 produced distinct clustering of physiological, neural, and behavioral effects in male and female mice, suggesting that emulsifier treatment leads to a syndrome of sex-dependent changes in

microbiota, physiology, and behavior. This study reveals that these commonly used food additives may potentially negatively impact anxiety-related and social behaviors and may do so *via* different mechanisms in males and females.

3.2 Introduction

The notion that the viscera or gut influences our emotions dates back over 100 years (James, 1884), and recent studies suggest this influence may be related to pathology. Indeed, a high comorbidity exists between gastrointestinal and psychiatric illnesses (Finegold et al., 2010; Adams et al., 2011; Dinan et al., 2014). An emerging focus of the gut-brain axis is the intestinal microbiota, the large and diverse community of microbes that reside in the gut, which has been shown to influence anxiety-like and social behaviors in mice. For example, mice reared in the absence of microbiota (germ-free mice) show lower anxiety-like behavior than conventionally-colonized mice (Diaz Heijtz et al., 2011; Neufeld et al., 2011b; Clarke et al., 2013), and introducing microbiota around the time of weaning partially normalizes anxiety-like behaviors (Bercik et al., 2011a; Diaz Heijtz et al., 2011; Neufeld et al., 2011a; Clarke et al., 2013). In addition, germ-free mice show reductions in social behavior (Desbonnet et al., 2014), and early life exposure to antibiotics also affects anxiety-like and social behaviors (Leclercq et al., 2017). Oral exposure to pathogenic bacteria increases the number of pro-inflammatory bacteria strains in the gut, and gastrointestinal inflammation increases anxiety- and depression-like behaviors in mice (Lyte et al., 1998; Lyte et al., 2006; Goehler et al., 2008; Bercik et al., 2010). In contrast, probiotics, which are anti-inflammatory (Rodes et al., 2013), reduce such behaviors (Bercik et al., 2010; Desbonnet et al., 2010; Bercik et

al., 2011b; Bravo et al., 2011; Jang et al., 2017a). Prebiotics, which act as food sources for anti-inflammatory microbiota, (Monteagudo-Mera et al., 2016) also reduce anxiety- and depression-like behaviors in mice (Burokas et al., 2017).

One potential mechanism for influencing intestinal microbiota, and thereby the inflammatory state, is through diet. Western diet is high in sugar, fats, red meats, refined grains, and processed foods containing food additives for both preservation and flavor and/or texture enhancement (Hu, 2002; Broussard and Devkota, 2016). Adding carboxymethylcellulose (CMC) or polysorbate-80 (P80) to the diet, commonly used emulsifying food additives, induces low-grade inflammation, obesity, and metabolic abnormalities in mice (Chassaing et al., 2015; Chassaing et al., 2017). The same treatments also promote microbial encroachment within the intestinal mucus barrier and alter microbiota species composition toward a more pro-inflammatory potential. Germ-free animals are protected from intestinal inflammation and metabolic abnormalities following emulsifier exposure, and transplant of microbiota from emulsifier-treated animals to germ-free recipient mice is sufficient to confer metabolic alterations, indicating that microbiota drive this phenotype (Chassaing et al., 2015). Taken together, these data further support the concept that microbiota composition is important for health, and that perturbations of the intestinal microbiota by modern stressors, such as emulsifiers, can lead to aberrant physiology.

In the present study, we examined the effects of emulsifier consumption on brain and behavior. We found that emulsifier treatment altered anxiety-like and social behaviors, as well as neuropeptide systems implicated in these behaviors, and did so in a sex-specific manner. Such sex-specific differences were paralleled by emulsifiers

having sex-specific effects on microbiota composition, inflammation and metabolism. These results demonstrate the potential for food additives that impact microbiota to broadly impact physiology and behavior.

3.3 Materials and Methods (Excerpt from original publication)

3.3.1 Animals

C57Bl/6J dams with litters (3 male and 3 female 14-day-old pups) were purchased from Charles River Laboratories. Mice were housed in ventilated transparent OptiMouse plastic cages with Bed-O-Cobs® and AlphaDri bedding (35.6 x 48.5 x 21.8cm; at Georgia State University). Lights were set to a 14h:10h light:dark cycle (lights off at 0900 ET), and ambient temperature was maintained at 23°C. Food (Purina rodent chow no. 5001) and water were available *ad libitum*. On postnatal day 21 (P21), mice were weighed and placed in a plastic container for approximately 20 minutes to collect feces for later analysis. Mice were put into a new cage such that each experimental group contained mice from all litters and that each litter was used for all experimental groups (Figure 1). Cages were given reverse-osmosis treated Atlanta city drinking water with sodium carboxymethylcellulose (CMC; Sigma, St. Louis, MO), or with polysorbate-80 (P80; Sigma) (1% in each case), or with no additives. The drinking water and emulsifier solutions were changed weekly. Body weights were measured weekly and expressed relative to the body weight on P21. All procedures were in accordance with the Guide for Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee at the Georgia State University (protocol number A15002).

3.3.2 Behavioral Testing

Starting on P70, behavioral tests were conducted once a week for 6 weeks, with a week between each test (Supplemental Figure 1). Behavioral tests were conducted in the following order: Open Field Test, Elevated Plus Maze, Light/Dark Box, Marble Burying Task, Three-Chambered Sociability Task, and Porsalt Forced Swim Test. Behavioral testing occurred within the first 4h after the start of the dark phase and was conducted under dim red light except for the Light/Dark box, which was illuminated by overhead lights (between 300-400 lux). Arenas were cleaned with 70% ethanol between trials. Behavioral tests were videotaped using a Sony camcorder for later analysis by AnyMaze version 4.96 (Stoelting, Co., Wood Dale, IL) or The Observer version XT11 (Noldus Information Technology Inc., Wageningen, The Netherlands). An experimenter blinded to the treatment conditions scored behavioral tests in the Observer.

3.3.2.1 Open Field Test

Locomotor behavior was assessed in a 43.2 X 43.2 X 30.5cm (WxLxH) Plexiglas arena (Med Associates, Inc., St. Albans, VT) containing 2 arrays of infrared transmitters strips (16 beams each) located on the bottom of the arena (in the X and Y plane). The center zone of the arena was defined as square containing the center 8 beams (e.g., beams 4-12) in the X and Y plane. Each mouse was placed into the arena with its nose facing the wall and allowed to freely investigate for 10 min. The total distance traveled, the total time spent in the center of the arena, and stereotypic circling behavior were calculated by Activity Monitor (Med Associates, Inc.,) on a computer connected to the open field arenas.

3.3.2.2 Elevated Plus Maze

An elevated plus maze with two open and two closed arms was used. Arms were 10 cm W X 50 cm L, connected by a 10 X 10 cm^2 center chamber. Closed arms had 40 cm H walls. The maze was elevated 50 cm from the floor. Mice were placed in the center of the arena and allowed to explore for 5 min. All trials were video-recorded from a digital camera mounted above the maze and connected to a computer. The number of entries into and the total time spent in the open arms, closed arms, or center were quantified by AnyMaze.

3.3.2.3 Light/dark Box

A 14.5 cm W X 30 cm L X 14 cm H chamber divided into a light and dark compartment was used. The light compartment (20 cm L) was made of white acrylic, and the dark compartment (10 cm L) of opaque black acrylic and covered. An opaque insert with a 5cm W X 5cm H opening connected the compartments. Mice were placed in the light compartment facing away from the entry into the dark chamber and allowed to freely investigate the chamber for 5 min. All trials were video recorded from a digital camera mounted above the Light/Dark box and connected to a computer. The number of entries into the dark chamber and total time spent in the light compartment were quantified in AnyMaze.

3.3.2.4 Marble Burying

A Plexiglas arena (24cm W X 46 cm L) was filled 4 cm deep with Alpha-dri bedding (Shepherd Specialty Paper, Fibercore, Cleveland, OH, USA). Mice were placed

into the arena, and after a 5 min habituation period, mice were removed and twenty marbles (17 mm) were evenly spaced on top of the bedding. Mice were placed in the center of the arena and video-recorded for 10 min. The number of marbles buried, as defined by being ½ or more covered with bedding, and the latency to bury the first marble were quantified using the Observer.

3.3.2.5 Three Chambered Sociability

A 24 X 74 X 24 cm (L x W x H) polycarbonate apparatus was divided into three equally sized chambers with openings 9cm W to allow free movements between compartments. At either end of the apparatus was an (9cm W X 10 cm H) opening beside which the stimulus cages were placed. The stimulus cages were 10cm W X 10 cm L X 10 cm H polycarbonate cage with grid (10 X 10) of small holes 0.5cm in diameter to allow transfer of visual and olfactory cues, while limiting physical interaction to nose contact or whisking.

3.3.2.6 Sociability test

Following a 5 min habituation period in which the mouse was allowed to explore the entire three-chambered apparatus, the experimental animal was removed, and an unfamiliar sex- and age-matched C57Bl6/J mouse was placed inside one of the stimulus cages beside one of the side chambers. An identical stimulus cage containing a novel object was placed beside the opposite chamber. The test animal was returned to the middle chamber and allowed to freely investigate the apparatus for 10 min.

The location of the novel mouse and the novel object were alternated between left and right chambers on consecutive sessions. The time spent and the numbers of entries into each chamber were measured using AnyMaze. The time spent sniffing or actively investigating the stimulus chambers over the 10 min test was scored in The Observer. A preference score was calculated by dividing the time spent investigating the novel mouse by the total time spent investigating the novel mouse and the novel object.

3.3.2.7 Social Preference test

Immediately following the 10 min sociability test, the experimental mouse was removed from the three-chambered apparatus, and the novel object was replaced with an unfamiliar stimulus sex- and age- matched C57Bl6/J mouse. The original stimulus mouse used in the sociability portion of the test remained in its cage beside one chamber of the apparatus. Identical measures were scored as in the sociability test: time spent in each chamber, entries between chambers, and time spent investigating each stimulus mouse.

3.3.2.8 Porsolt Forced Swim Test

A vertical Plexiglas cylinder (40cm H X 18cm diameter) was filled with 3L of 30 °C (± 2 °C) water. Mice were placed in the center of the cylinder and video recorded for 5 mins. The latency and duration of immobility were quantified in the Observer. Immobility was defined by the absence of movement or only small movements of posterior paws that did not result in displacement of the water. At the end of the test,

mice were removed from the cylinder and placed in a recovery cage on a heating pad until they were dry and then returned to their home cage.

3.3.3 Euthanasia and Tissue Collections

One day following completion of behavioral testing (P105), mice were deeply anesthetized under isoflurane (5%v/v) and body weight was recorded. Blood was collected from the retrobulbar intraorbital capillary plexus. Mice were euthanized by cervical dislocation, and the colons, spleens, livers, adipose, feces, and brains were collected for subsequent analysis. Hemolysis-free serum was generated by centrifugation blood samples using serum separator tubes (Becton Dickinson, Franklin Lakes, NJ). The weight and length of the colon and weights of the spleen, liver, and perigonadal adipose fat depot were recorded and normalized to the body weight. Brains were removed and fixed in a 5% acrolein in sodium phosphate buffer (0.1M, pH 7.4) at 4°C, followed by cryoprotection in 30% sucrose in phosphate buffered saline (PBS: 0.05M, ph7.4).

3.3.4 Immunohistochemistry

Brains were sectioned (30µm) in the coronal plane with a cryostat and stored in a cryoprotectant solution (ethylene glycol/sucrose in sodium phosphate buffer) until immunostained. Free-floating sections were rinsed three times in Tris-buffered saline (TBS; 0.05 M Tris, 0,9% NaCl, pH 7.6), then incubated for 30 min in 0.05 M sodium citrate in TBS. After rinsing in TBS sections were places for 30 min in 0.1 M glycine in TBS, rinsed again, and placed into blocking solution (10% normal goat serum (NGS),

0.4% Triton-X and 1% H2O2 in TBS) for 30 min. Sections were incubated overnight in one of the following primary antibodies diluted in 2% NGS and 0.4% Triton-X in TBS: anti-vasopressin (Bachem; 1:32000) anti-oxytocin (Peninsula Labs; 1:32000); anti-agouti-related peptide (AgRP; Phoenix Pharmaceuticals; 1:250000), anti-alpha-melanocyte stimulating hormone (MSH; Millipore; 1:100000), and anti-ionized calcium-binding adaptor protein (Iba1; Wako Laboratoy; 1:30000). The next day, sections were rinsed three times in TBS containing 1% NGS and 0.02% Triton-X and incubated in biotinylated secondary antiserum [goat anti-rabbit for vasopressin, oxytocin, AgRP, and Iba1 immunoreactivity; rabbit anti-sheep for MSH (Vector Laboratories, Burlingame, CA)] diluted 1:800 in TBS with 2% NGS and 0.32% Triton-X for 1 h. This was followed by rinses in TBS containing 0.4% Triton X, incubated in avidin-biotin complex (Vectastain Elite ABC Kit; Vector Laboratories) diluted to 1:800 in TBS for 1 h, followed by three TBS rinses and three sodium acetate buffer rinses. Finally, the staining was visualized using nickel-enhanced diaminobenzidine (DAB) Substrate Kit (Vector Laboratories). Sections were mounted onto gelatin-coated slides and cover-slipped with Permount.

3.3.5 Image Analysis

Slides were anatomically-matched and analyzed by an investigator blinded to the experimental groups. Sections were imaged using a Zeiss Axio Imager M2 microscope connected to an ORCA-R2 CCD digital camera (Hamamatsu Photonics). Gray-scale images of the fiber density in the photomicrographs were analyzed in Image J 1.43u (National Institutes of Health, Bethesda, MD). The region of analysis was outlined in

each section. Subjects for which the relevant sections were damaged or unavailable were dropped from a given analysis.

3.3.6 Fecal microbiota 16s rRNA gene sequencing and sequences analysis

16S rRNA gene amplification and sequencing were done using the Illumina MiSeq technology following the protocol of Earth Microbiome Project with their modifications to the MOBIO PowerSoil DNA Isolation Kit procedure for extracting DNA (www.earthmicrobiome.org/emp-standard-protocols) (Gilad et al., 1987; de Paz Cabello et al., 1988). Bulk DNA was extracted from feces collected on P21 and P105 using a PowerSoil-htp kit from MoBio Laboratories (Carlsbad, CA, USA) with mechanical disruption (bead-beating). The 16S rRNA genes, region V4, were PCR amplified from each sample using a composite forward primer and a reverse primer containing a unique 12-base barcode, designed using the Golay error-correcting scheme, which was used to tag PCR products from respective samples (de Paz Cabello et al., 1988). We used the forward primer 515F 5'-*AATGATACGGCGACCACCGAGATCTACAC***TATGGTAATTGT**GTGCCAGCMGCCGCGGTAA-3': the italicized sequence is the 5' Illumina adapter B, the bold sequence is the primer pad, the italicized and bold sequence is the primer linker and the underlined sequence is the conserved bacterial primer 515F. The reverse primer 806R used was 5'-*CAAGCAGAAGACGGCATACGAGAT* XXXXXXXXXXXX **AGTCAGTCAG CC** GGACTACHVGGGTWTCTAAT-3': the italicized sequence is the 3' reverse complement sequence of Illumina adapter, the 12 X sequence is the golay barcode, the bold sequence is the primer pad, the italicized and bold sequence is the primer linker and the

underlined sequence is the conserved bacterial primer 806R. PCR reactions consisted of Hot Master PCR mix (Quantabio, Beverly, MA, USA), 0.2 μM of each primer, 10-100 ng template, and reaction conditions were 3 min at 95°C, followed by 30 cycles of 45 s at 95°C, 60s at 50°C and 90 s at 72°C on a Biorad thermocycler. PCRs products were purified with Ampure magnetic purification beads (Agencourt, Brea, CA, USA), and visualized by gel electrophoresis. Products were then quantified (BIOTEK Fluorescence Spectrophotometer) using Quant-iT PicoGreen dsDNA assay. A master DNA pool was generated from the purified products in equimolar ratios. The pooled products were quantified using Quant-iT PicoGreen dsDNA assay and then sequenced using an Illumina MiSeq sequencer (paired-end reads, 2 x 250 bp) at Cornell University, Ithaca.

Forward and reverse Illumina reads were joined using the fastq-join method (Aronesty, 2011, 2013), sequences were demultiplexed, quality filtered using Quantitative Insights Into Microbial Ecology (QIIME, version 1.8.0) software package (Giraldo et al., 1985). QIIME default parameters were used for quality filtering (reads truncated at first low-quality base and excluded if: (1) there were more than three consecutive low quality base calls (2), less than 75% of read length was consecutive high-quality base calls (3), at least one uncalled base was present (4), more than 1.5 errors were present in the bar code (5), any Phred qualities were below 20, or (6) the length was less than 75 bases). Sequences were assigned to operational taxonomic units (OTUs) using UCLUST algorithm (Edgar, 2010) with a 97% threshold of pairwise identity (without the creation of new clusters with sequences that do not match the reference sequences), and classified taxonomically using the Greengenes reference database 13_8 (Cedar, 1988). A single representative sequence for each OTU was

aligned and a phylogenetic tree was built using FastTree (Cowing et al., 1989). The phylogenetic tree was used for computing the unweighted UniFrac distances between samples (Bergman et al., 1992; Zembrzuski et al., 1992). rarefaction were performed and used to compare abundances of OTUs across samples. Principal coordinates analysis (PCoA) plots were used to assess the variation between experimental group (beta diversity). Alpha diversity curves were determined for all samples using the determination of the number of observed species. LEfSE (LDA Effect Size) was used to investigate bacterial members that drive differences between groups (Day et al., 1988). The threshold on the logarithmic LDA score for discriminative features was set to 2.0, and the alpha values for the factorial Kruskal-Wallis test and pairwise Wilcoxon test between subclasses were set to 0.05.

In addition to fecal samples collected from the animals used in this current study, the 16s sequences previously generated (from Chassaing et al., 2015) were reanalyzed by combining gene sequences from both male and female mice treated with water (male: 12; female: 12), CMC (male: 11; female: 12), and P80 (male: 10; female: 9).

3.3.7 Statistical analyses

Data were analyzed using IBM SPSS Statistics Version 21 (IBM) and visualized using GraphPad Prism 7.0c (GraphPad Software, La Jolla, CA). Body weights were analyzed by a repeated measure ANOVA, with sex and treatment as factors, followed by Fishers' LSD as post hoc analyses. Anxiety-like and social behaviors were analyzed by a two-way ANOVA with treatment and sex as the factors, followed by Fishers' LSD as post hoc analyses.

Data were also analyzed by multiple discriminant analysis (MDA) in order to reveal patterns in the aggregate behavioral changes. Discriminant analysis is a multivariate data analysis technique that employs algorithms used in machine learning to reveal the combination of measures that best differentiate sample groups. These analyses used a wide array in input variables in order to capture syndromic treatment effects across the various experiments. Here, we use it to explore the combination of locomotor, anxiety-like, and repetitive behaviors observed in the open field test, and also use a select few measures that most intuitively capture locomotor, anxiety-like, and repetitive behaviors (e.g. time spent moving, time in center of open field, and time spent in stereotypic circling) along with measures for hypothalamic neuropeptide expression and measures of metabolic state to explore larger systemic impact of emulsifier treatment on our subject mice. By convention, this algorithm returns five “discriminant functions”, each with a unique combination of weights for the input variables. These functions are ordered from those that describe most to the least of the variance in the data set. Wilk’s lambda is the statistical test that describes which of these successively ranked functions significantly differentiate the function groups. The weights of each of the measures along each of the discriminant functions is listed in structure matrices, which are referenced in the text. Any measure with a weight greater than 0.3 is considered to significantly contribute to the described discriminant function.

3.4 Results

3.4.1 Effects of emulsifiers on host physiology and metabolism

In accord with our previous work, twelve weeks of exposure to emulsifiers carboxymethylcellulose (CMC) or polysorbate (P80) via drinking water led to a marked increase in abdominal adiposity that was associated with chronic mild intestinal inflammation, as revealed by shorter colons and increased spleen weight (Fig. 3.1).

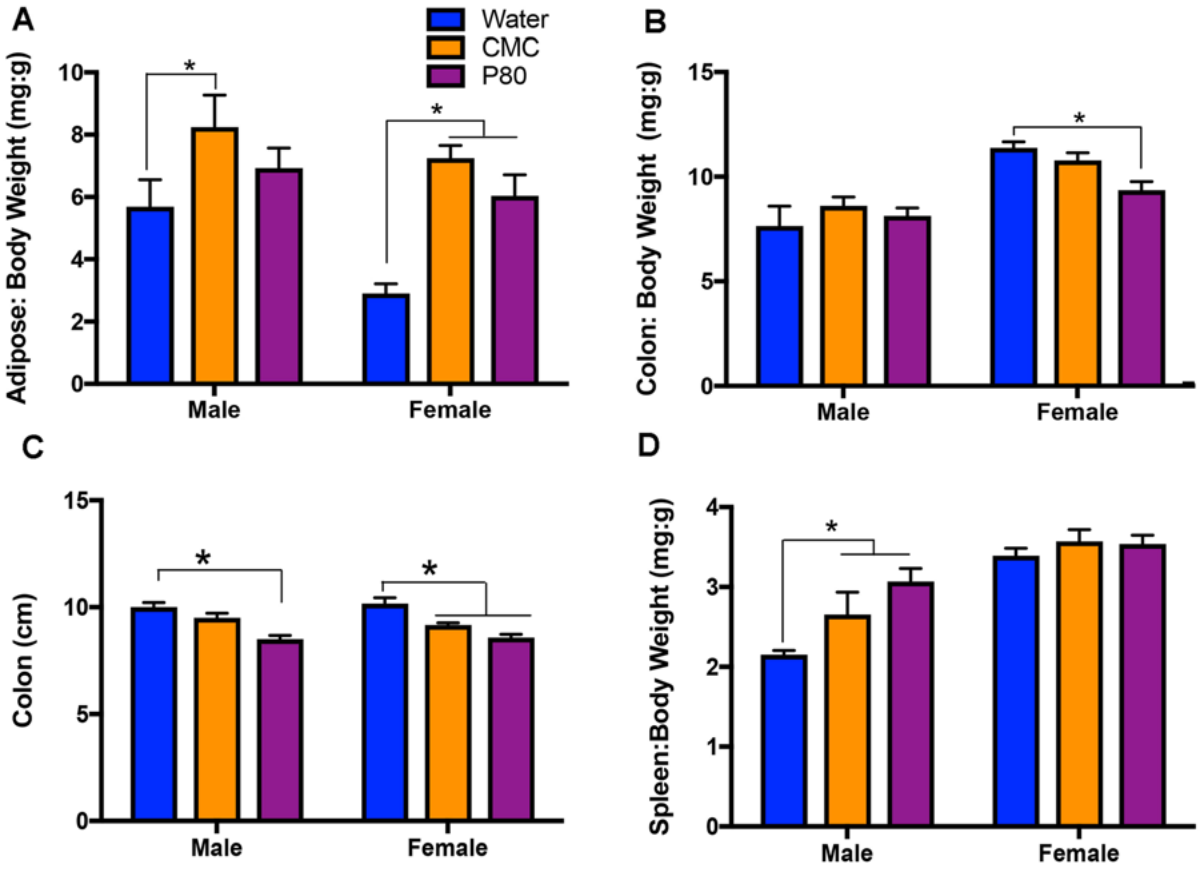

Figure 3.1 Dietary emulsifiers promote physiological changes consistent with metabolic syndrome.

Male and female C57Bl/6 mice were exposed to drinking water containing CMC or P80 (1%) for 12 weeks. ***(A)*** *There was a main effect of treatment with emulsifiers on*

*fat-pad mass [$F_{(2,29)}$ = 12.48, p < 0.001]. There is also a main effect of sex on adiposity such that males had greater fat mass than did females $F_{(1,29)}$ = 7.65, p < 0.01]. Post-hoc comparisons indicate that both CMC and P80 increased fat mass in females, but in males only CMC treatment increased fat mass compared to respective water-treated controls (*p < 0.05).* ***(B)*** *There was a significant interaction of emulsifier treatment and sex on colon weights [$F_{(2,29)}$ = 3.383, p < 0.05], with post-hoc comparisons indicating that P80 treatment significantly reduced colon weights in female compared to water-treated controls (*p < 0.05).* ***(C)*** *Emulsifier treatment also had a significant main effect on colon lengths in male and female mice [$F_{(2,29)}$ = 28.70, p < 0.0001] such that females treated with both emulsifiers and males treated with P80 had significantly shorter colons compared to their respective water-treated controls (*p < 0.05).* ***(D)*** *There was a significant main effect of both emulsifier consumption [$F_{(2,29)}$ = 5.312, p < 0.05] and sex on spleen weights [$F_{(1,29)}$ = 43.31, p < 0.0001]. Post-hoc comparisons indicate that treatment with both CMC and P80 increased spleen weight compared to water-controls in males, but not females (*p < 0.05). Data are represented as means + SEM (n = 5–6).*

3.4.2 Impact of emulsifier consumption on fecal microbiota composition

We next used 16S rRNA sequencing to determine the effects of emulsifier consumption on microbiota composition. Using Principal Coordinate Analysis (PCoA) of the unweighted UniFrac distances, we first examined the differences in microbiota composition before treatments begin (P21). As expected, the microbiota did not differentially cluster prior to treatment (Fig. 3.2A,B). In addition, LefSe analyses identified very few operational taxonomic units (OTUs) with an altered abundance

between treatment (Supplemental Fig. 2A,B,E,F). Importantly, following twelve weeks of emulsifier exposure (P105), male and female animals harbored distinct microbiota composition based on treatment (Fig. 3.2C,D, Permanova < 0.001). LefSe analysis conducted in males and females separately indicated that several taxa differ based on treatment: in males, emulsifier comsumption reduced the abundance of the Firmicutes phylum and *Oscillospria*, *Coprococcus*, and *rc4_4* genera (Supplemental Fig. 2C,D). CMC-treated males exhibited higher abundance of the genus *Dorea* whereas P80-treatment increased the abundance of the genera *Bacteroides*, *Burkholderia*, *Clostridium*, and *Veillonella*. In females, emulsifier treatment reduced abundance of *Bacteroides*, *Sphingomondales*, *Sphingomonas*, *and Ruminococcus* (Supplemental Fig. 2G,H). CMC-treated females showed increases in *Anaeroplasma*; whereas P80 treatment increased the relative abundance of the Proteobacteria phylum and of *Clostridium* and *Burkholderia* genus.

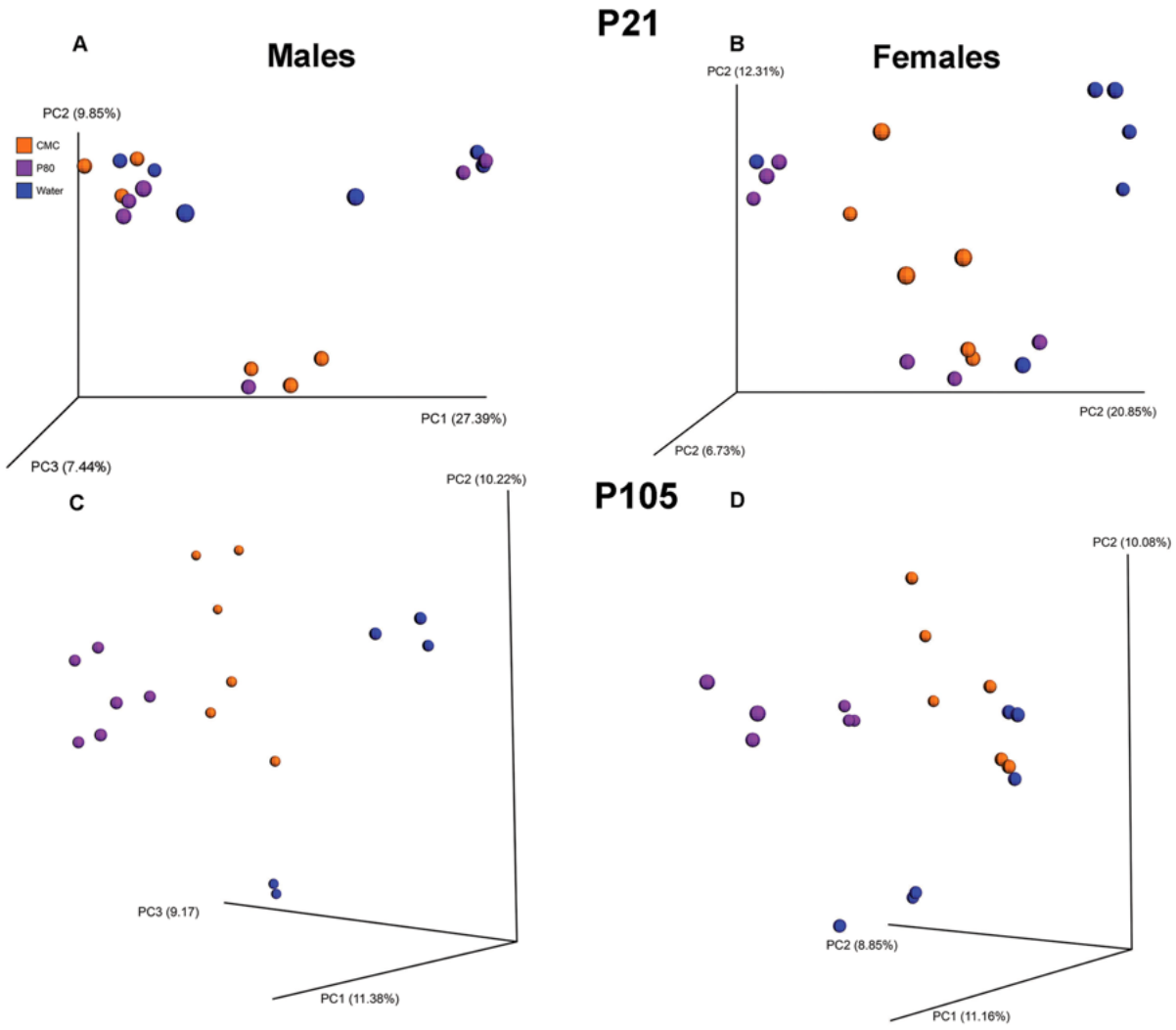

Figure 3.2 Effects of dietary emulsifiers on microbiota.

*Principal coordinates analysis (PCoA) of the unweighted UniFrac distance matrix of fecal microbiota in male (**A**,**C**) and female (**B**,**D**) mice at the time of weaning, P21 (**A**,**B**) and at the time of collections, P105 (**C**,**D**). Treatment of the mouse is indicated by point color (blue, water; orange, CMC; purple, P80).*

We next analyzed microbiota composition in animals from the current study and in animals from our previous work (Fig. 3.3 and Supplemental Fig. 3) in order to examine sex differences in the microbial community structure (Chassaing et al., 2015).

Such analysis revealed an impact of sex on microbiota composition, for each experimental group (water, CMC and P80, Fig. 3.3). When each treatment group was examined separately, there were sex differences in community composition. (Fig. 3.3). For example, within the water-treated controls, bacteria from genera *Bacteroides* and *Clostridium* were more abundant in females, whereas bacteria within the genera *Lactobacillus* and *Coprococcus* were more highly present in males. (Fig. 3.3B,D).

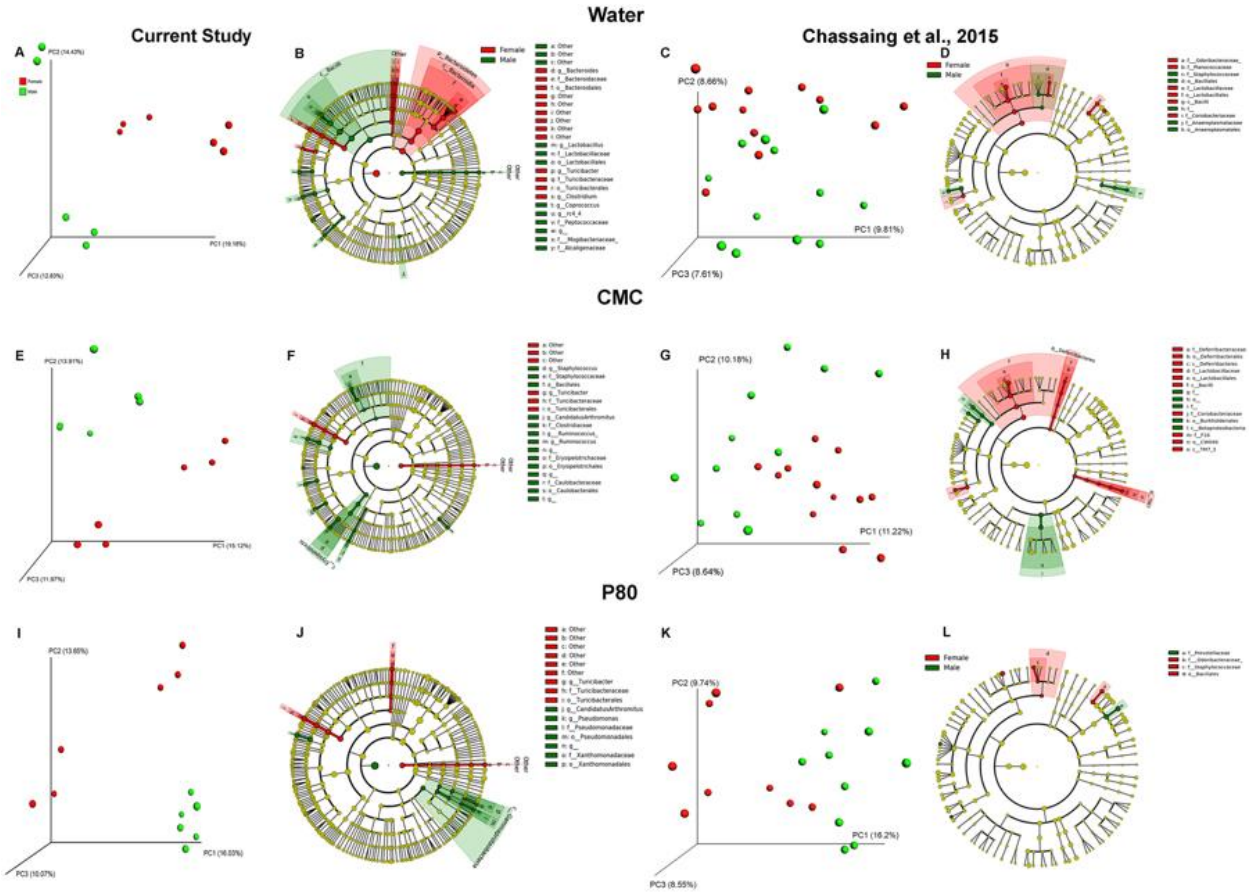

Figure 3.3 Sex differences in microbiota in mice treated with dietary emulsifiers.

Male and female C57Bl/6 mice were exposed to drinking water containing CMC or P80 (1%) in the present study and in data previously reported in Extended Data Fig.

*3 in (Chassaing et al., 2015). Principal coordinates analysis (PCoA) of the unweighted UniFrac distance matrix of fecal microbiota showing clustering by sex in (**A**,**C**) water-, (**E**,**G**) CMC- and (**I**,**K**) P80- treated mice. Sex of the mice is indicated by point color (red, female; green, male). Linear discriminant analysis coupled with Effect Size (LEfSe) was used to identify taxa that differ significantly between male and female mice within water, CMC, and P80 treatments.*

Treatment with emulsifiers changed the gut microbiota of males and females differently (Fig. 3.3). For example, the sex differences in the genera *Bacteroides*, *Closteridium*, *Lactobacillus* and *Coprococcus* are eliminated following CMC treatment. Some new sex differences also emerged: following CMC treatment in males, an increased abundance in bacteria pertaining to the genera *Staphylococcus* and *Ruminococcus* (Fig. 3.3F) was observed, and females harbored more bacteria within the phyla *Deferribacteres* and *TM7* (Fig. 3.3H). Following P80 treatment, males displayed an increased abundance of the genus *Pseudomonas* (Fig. 3.3J,L).

3.4.3 Effects of emulsifiers on behavior

3.4.3.1 Anxiety-like behavior - Open Field Test

We next sought to determine the impact that emulsifier consumption and associated alterations in microbiota composition might have on behavior. We observed that, in male animals, emulsifier treatment reduced the time spent in the center portion of the open field (Fig. 3.4A) without affecting the total distance traveled in the apparatus (Supplemental Fig. 4A). In addition, there was a trend towards a main effect of sex, such that females spent less time in the center, compared to males ($p = 0.07$; Fig. 3.4A), mostly driven by the time spent in the center in the male water group. Multivariate test statistics revealed that the behaviors in the open field test separated significantly by sex and emulsifier treatment along five discriminant functions (Table 1). Function 1 explained 43.9% of the variance in the data set ($R = 0.898$) and the number of stereotypic beam breaks maps most highly onto this function ($r = 0.383$). The canonical discriminant function plot reveals the effects of each individual emulsifier treatment on each of the sexes for these two functions in the open field behaviors. Emulsifier consumption causes a separation of the aggregate open field behaviors from the water-treated controls. Moreover, the changes in the open field behaviors are similar in P80-treated male and female mice, whereas, CMC may exert unique effects in male and females (Fig. 3.4B).

While focusing on the unique behavioral effects of each emulsifier treatment across each sex, we chose to examine several different measures from the open field test. Here, we used MDA across 15 different measures: locomotor (resting time, average velocity, ambulatory episodes, ambulatory time, ambulatory counts, and

ambulatory distance), repetitive (stereotypic counts, time spent in stereotypic movement, jump counts, jump time, clockwise reversals, and counterclockwise reversals), and anxiety-like (time-spent in center zone, number of entries into the center zone, and time spent in vertical posture). Here, again, we observe that while P80 largely maintains sex differences demonstrated in water-treated mice along function 1, CMC uniquely affects sex differences across this syndrome of behavioral measures. In addition, sex differences are not observed for water-treated mice along function 2, however here, P80 and CMC appear to only exert their effects in one given sex each: whereas P80 affects females along function 2 most robustly, CMC exerts its greatest effects in males. In addition, CMC and P80 appear to exert similar effects within their affected sex along function 2. The measures that map on best to function 1 are repetitive and locomotor measures, namely (by order of significance) stereotypic counts, resting time, average velocity, jump counts, and stereotypic time. The measures that map on best to function 2 are locomotor, anxiety-like, and repetitive measures, namely (by order of significance) ambulatory episodes, number of entries into the center zone, clockwise reversals, jump time, and jump counts. Thus, while P80 does not as robustly affect sex differences in patterns of general locomotor and repetitive movements (observed in water-treated mice), CMC does disrupt, and perhaps invert, these sex differences. In addition, when anxiety-like measures are more fully represented (in discriminant function 2), it is clear that each emulsifier has a unique effect on males and females, each one mostly affecting one or the other sex.

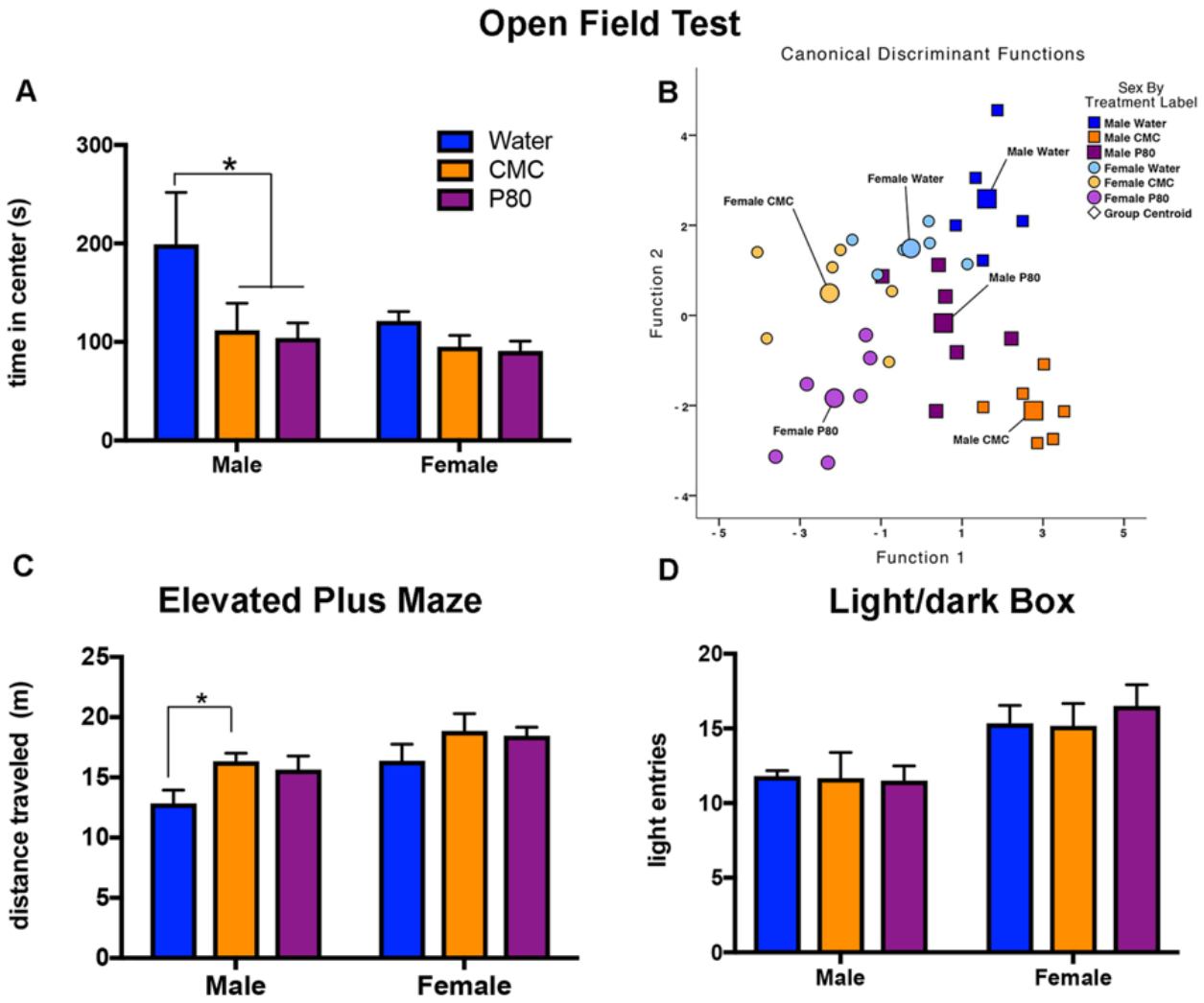

Figure 3.4 Dietary emulsifiers alter anxiety-like behaviors in male and female mice.

*(**A**) There was a main effect of emulsifier treatment to decrease the time spent in the center of the open field test [$F_{(2,29)} = 4.14$, $p < 0.05$]. Post-hoc analyses indicate that in males, treatment with emulsifiers decreases in the time spent in the center, compared to water-treated controls (*$p < 0.05$). (**B**) Multivariate test statistics measured the impact of additional behavioral measures captured in the automated open field apparatus (Table 1). The canonical discrimination function plot and Wilk's lambda revealed a significant separation of groups by sex and emulsifier consumption along five*

*discriminant functions [$\Lambda = 0.010$, $X^2(30) = 108.204$, $p < 0.01$]. (**C**) Emulsifier consumption increased the total distance traveled in the elevated plus maze [$F_{(2,29)} = 3.94$, $p < 0.05$]. Post-hoc analyses indicate that in males, treatment with CMC significantly increased the distance traveled in the EPM (*$p < 0.05$). In addition, there was a main effect of sex [$F_{(1,29)} = 10.42$ $p < 0.01$], such that females traveled a greater distance, compared to males, regardless of treatment. (**D**) There was no significant effect of emulsifier treatment on the total number of entries into the light portions of the light/dark box. Irrespective of treatment, however, female mice made significantly more entries into the light portion of the light/dark box [$F_{(1,29)} = 13.76$, $p < 0.001$]. Data are represented as means + SEM ($n = 5–6$).*

Table 3.1 Structure Matrix of Discriminant Analysis for Open Field Behavior.

Measured Outcomes	Function				
	1	2	3	4	5
Circling Counts	.383*	.118	-.119	.053	.153
Resting Time (sec)	.298*	.186	-.023	.160	.214
Time in Center Zone (sec)	.147*	-.109	-.104	.145	-.091
Number of Entries into Center Zone	.155	.262*	.051	.260	.159
Jump Counts	-.259	.213	.435*	.138	-.048
Average Velocity	-.295	.150	.304*	.190	-.164

Counter Clockwise Reversals	.057	-.127	.372	.381*	-.039
Ambulatory Episodes	.080	.280	-.071	.304*	-.021
Vertical Time (sec)	.062	-.060	-.159	.256*	-.141
Jump Time (sec)	-.007	.235	.112	.043	.450*
Circling Time (sec)	-.210	-.066	.205	.022	.406*
Ambulatory Time (sec)	-.144	-.090	.181	.057	.401*
Ambulatory Counts	-.179	-.127	.202	-.003	.389*
Ambulatory Distance (cm)	-.115	-.053	.165	.005	.345*
Clockwise Reversals	-.032	.242	.087	-.179	.317*

*Pooled within-group correlations between discriminating variables and standardized canonical discriminant functions. The variables are ordered by absolute size of correlation within each the functions (*indicates the largest absolute correlation between each variable and any discriminant function).*

3.4.3.2 Anxiety-like behavior - Elevated Plus Maze Test

Treatment with emulsifiers did not affect time spent in, nor number of entries into, either the open or closed arms in the elevated plus maze test (Supplemental Fig. 4). Emulsifier consumption did, however, increase the distance travelled in this behavioral test (Fig. 3.4C), suggesting that although emulsifier consumption did not increase anxiety in this test, it impacted locomotor behavior.

3.4.3.3 Anxiety-like behavior - Light/Dark Box

Treatment with emulsifiers did not affect time spent in the light nor the number of entries into the light (Fig. 3.4D and Supplemental Fig. 4F), suggesting that emulsifiers did not impact anxiety in the light/dark box test. Irrespective of treatment, female mice made significantly more entries into the light, but the total amount of time spent in the light did not differ from male mice (Fig. 3.4D).

3.4.3.4 Anxiety-like behavior - Marble Burying Task

Treatment with emulsifiers did not significantly affect the number of marbles buried ($p=0.91$; data not shown) or the latency to bury the first marble ($p=0.69$; data not shown).

3.4.3.5 Sociability

Treatment with emulsifiers did not affect social interaction as measured by the percent of time spent investigating a novel mouse when given the choice to investigate

that mouse or a novel object (Fig. 3.5A). However, if given a choice between a novel or a familiar mouse, emulsifier treatment lowered the preference for the novel mouse compared to water-treatment in females (Fig. 3.5B). Indeed, post-hoc comparisons indicated that CMC consumption in female mice significantly reduced the preference for the novel mouse. In addition, there was a strong trend towards a reduced preference for the novel mouse following P80 consumption in female mice ($p = 0.06$) (Fig. 3.5).

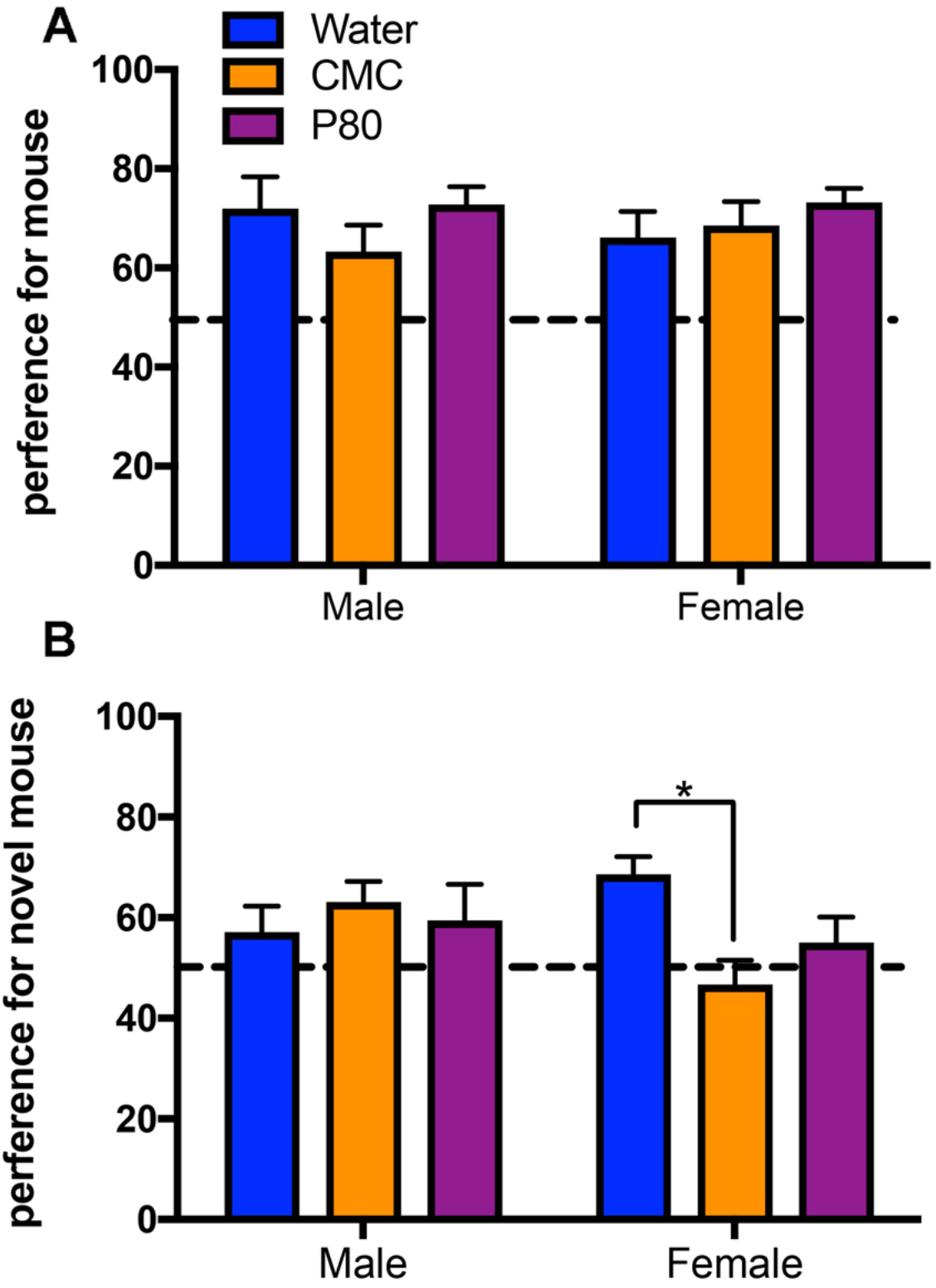

Figure 3.5 **Dietary emulsifiers decrease preference for social novelty in female mice.**

*(**A**) There was no significant main effect of emulsifier consumption [$F_{(2,28)}$ = 1.08, p = 0.35] or sex [$F_{(1,28)}$ = 0.00003 p = 0.99] on the preference for investigating a novel, conspecific mouse during the sociability test in the three-chambered sociability apparatus. In addition, there was no sex by treatment interaction on this measure [$F_{(2,28)}$ = 0.67, p = 0.52] (**B**). Emulsifier treatment and sex interacted on the preference for investigating a second novel, conspecific mouse during the preference for social novelty test in the three-chambered sociability apparatus [$F_{(2,29)}$ = 3.71, p < 0.05]. In addition, post-hoc comparisons indicate that treatment with CMC significantly decreased the preferences of female mice for the novel mouse (*p < 0.05). Data are represented as means + SEM (n = 5–6).*

3.4.3.6 Depression-like behavior

Treatment with emulsifiers did not affect the duration of immobility (p=0.92; data not shown) or the latency to first bout of immobility (p=0.30; data not shown).

3.4.3.7 Effects of emulsifiers on neural correlates

We next investigated the effects of emulsifiers on neuropeptides that influence feeding and anxiety behaviors. Both agouti-related peptide (AgRP) and α-melanocyte stimulating hormone (α-MSH) regulate appetite, energy homeostasis, and anxiety like behavior with AgRP stimulating food intake and reducing anxiety-like behaviors and αMSH acting in opposition to inhibit food intake and increase anxiety-like behaviors (Kokare et al.; Liu et al., 2007; Dietrich et al., 2015). In males, consumption of CMC increased AgRP immunoreactivity (IR) in the arcuate nucleus (Fig. 3.6A) and both CMC

and P80 consumption increased AgRP IR in the paraventricular nucleus of the thalamus of male animals (PVT; $p = 0.05$, Fig. 3.6B). Emulsifier treatment reduced α-MSH IR in the PVT of both male and female animals (Fig. 3.6C). Females also have increased αMSH IR compared to males in both the PVT and the arcuate nucleus ($p < 0.0001$; data not shown). Treatment with emulsifiers was without effect on αMSH IR in the arcuate nucleus ($p = 0.95$; data not shown) and the PVN ($p = 0.91$; data not shown).

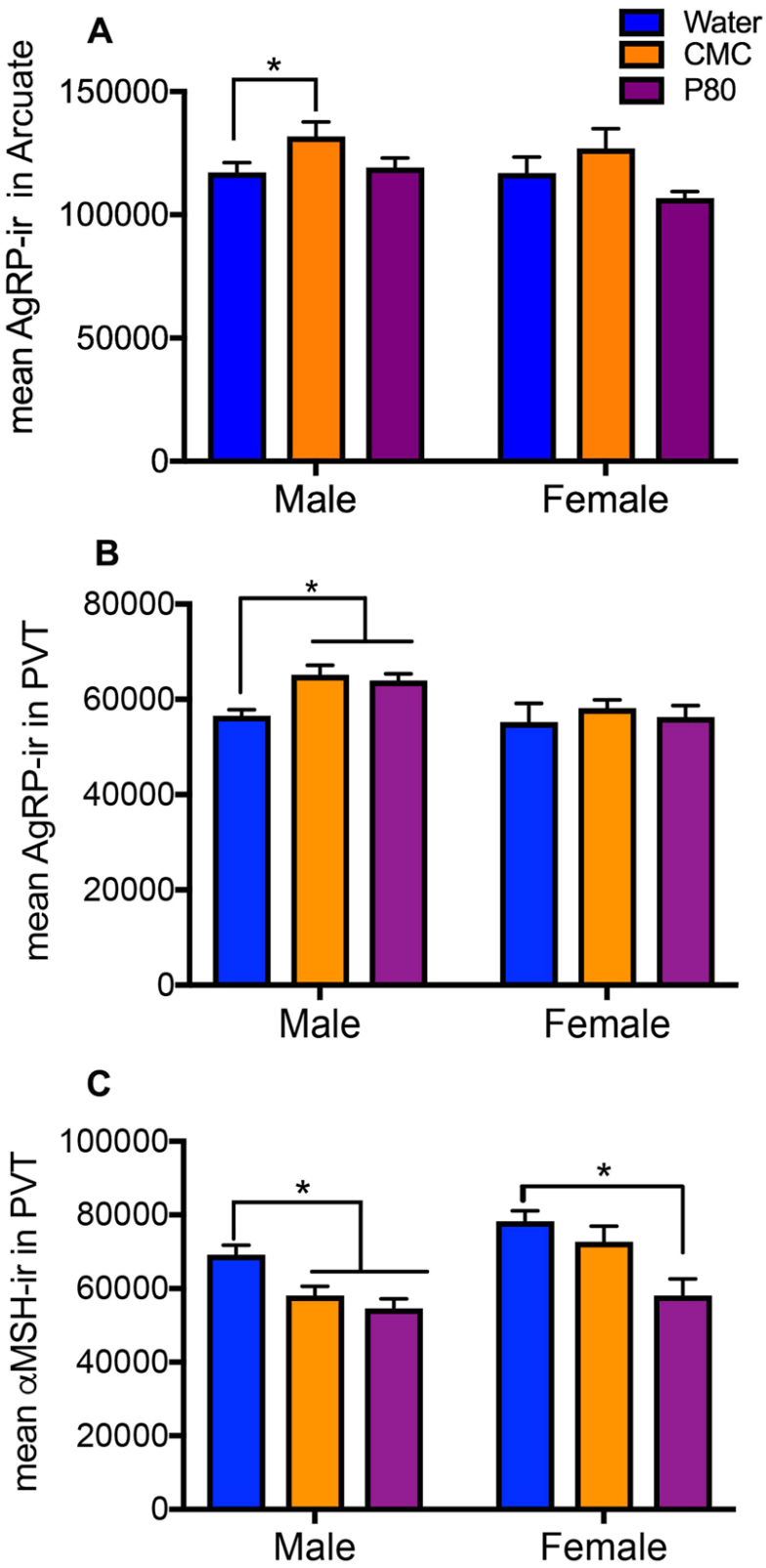

Figure 3.6 Dietary emulsifiers alter neuropeptide immunoreactivity in male and female mice.

*(**A**) There was a main effect of emulsifier consumption on agouti-related peptide (AgRP)-immunoreactivity (IR) in the arcuate nucleus in both male and female mice [$F_{(2,29)} = 4.689$, $p < 0.05$], driven, in part, by the significant increase following consumption of CMC in males (*$p < 0.05$). (**B**) There was a main effect of sex on AgRP-IR in the paraventricular nucleus of the thalamus (PVT) [$F_{(1,29)} = 7.494$, $p < 0.05$], such that males had more AgRP-IR than females. In addition, post-hoc analysis indicated that consumption of emulsifiers significantly increased AgRP-IR in the PVT of males (*$p < 0.05$). (**C**) Emulsifiers reduced α-Melanocyte Stimulating Hormone (αMSH)-IR in the PVT in male and female mice [$F_{(2,29)} = 12.98$, $p < 0.001$]. In addition, there was also a main effect of sex with females having more αMSH-IR than males [$F_{(1,29)} = 14.42$, $p < 0.001$]. Data are represented as means + SEM ($n = 5–6$).*

That dietary emulsifiers induced chronic intestinal low-grade inflammation led us to examine whether emulsifier altered microglia by examining Iba1-immunoreactivity. Emulsifier treatment did not affect total Iba1-immunoreactivity in the PVT, PVN, Arcuate nucleus, or hippocampus (data not shown), suggesting that emulsifier consumption does not lead to gross changes in microglia.

3.4.3.8 Multivariate analysis of emulsifier effects

Next, multivariate tests were used in order to measure the impact of emulsifiers on synergistic changes in the behavioral measures, physiological parameters, and immunoreactivity of neuropeptides in the PVT. When these measures were analyzed in combination, Wilk's lambda revealed a significant separation of groups by sex and emulsifier treatment along five discriminant functions. The variables that map most

highly onto these functions are listed in Table 3.2. Functions 1 and 2 explain the majority of variance in the data set (cumulatively, 80.0% of the variance), with Function 1 explaining 53.8% of the variance (*R*=0.945) and Function 2 explaining 26.2% of the variance (*R*=0.896).

Table 3.2 Structure Matrix of Discriminant Analysis for Physiological, Behavioral, and Neuropeptidergic Effects of Emulsifier Treatment.

Measured Outcomes	Function				
	1	2	3	4	5
Relative Body Weight	.418*	-.088	-.098	-.242	.103
Colon Weight	-.102*	-.004	.072	.022	.005
MSH-IR in PVT	-.113	.520*	.209	-.374	-.013
Fat Pad Weight	.229	.231*	-.111	.163	.022
Stereotypic Counts	.044	.105	-.489*	.027	-.168
Time in Open Arm	.011	.008	.236*	-.185	-.058
Spleen Weight	-.081	-.082	.190*	-.129	.059
AgRP-IR in PVT	.076	-.264	-.220	-.358*	-.082
Number of Marbles Buried	.101	-.042	.093	-.269*	-.172
Time in Light	.059	-.045	.129	.265*	-.253
Social Preference	.050	.015	-.011	.262*	-.107
Social Interaction	.003	.084	.147	.169*	-.129

AVP-IR in PVT	.072	-.065	-.261	-.150	.504*
Time in Center Zone	.058	.095	-.039	.002	-.423*
Colon Length	.004	.021	.073	.046	.156*

*Pooled within-group correlations between discriminating variables and standardized canonical discriminant functions. The variables are ordered by absolute size of correlation within each the functions (*indicates the largest absolute correlation between each variable and any discriminant function).*

Canonical discriminant function plot reveals the effects of each individual emulsifier treatment on each of the sexes for these two functions (Fig 3.7). In males, emulsifier treatment separates along Function 1 with the group centroid for CMC treatment lower in value and the group centroid for P80 treatment greater in value than the centroid for the respective water-treated controls (Table 3.3). Furthermore, in females, both centroids for CMC and P80 treatment are greater in value than for water treatment. However, along Function 2, the group centroids for CMC and P80 are in the same direction with respect to the group centroids for water for both males and females. Altogether, these data demonstrate that CMC and P80 altered both physiology and behavior, with some differential effects in males and females.

While it is clear that both emulsifiers had a significant effect on both sexes across function 2, each emulsifier had a unique effect on sex differences in these measures weighted along function 1. While sex differences observed in water-treated controls were largely maintained with P80 treatment along function 1, some of these sex

differences appear to be reversed by CMC along this axis. Function 2 is most heavily weighted by a mixture of behavioral, physiological and neural measures, including (listed by significance) MSH and AgRP immunoreactivity in the paraventricular nucleus of the thalamus, perigonadal fat pad weight, time spent in the center zone, and body weight increase relative to initial weight. Function 1 is most heavily weighted by primarily physiological markers, including (listed by significance) body weight increase relative to initial weight, perigonadal fat pad weight, MSH immunoreactivity in the paraventricular nucleus of the thalamus, colon weight, and spleen weight. However, a broader mixture of measures contributed to this analysis, and all measures combine to give a full picture of the effects of these emulsifiers on the subject mice. The physiological measures included relative body weight, colon weight, fat pad weight, spleen weight, and colon length. The neural measures included MSH, AgRP and AVP immunoreactivity in the paraventricular nucleus of the thalamus. The behavioral measures included time in open arms of elevated plus maze, number of marbles buried, time in light portion of the light/dark box, social preference and social interaction time in three-chamber social interaction test, and time in center zone and stereotypic counts of the open field test. Hints at sexually differential effects of these measures were revealed with ANOVAs on individual measures. For example, only males demonstrated a relative increase in body weight with emulsifier treatment whereas females maintained the same weight throughout. However, both emulsifiers are observed here to increase relative body weight equally in males. Nevertheless, combined with observing other physiological measures, in concert with examining syndromic effects on neural markers

and behavioral output, CMC appears to affect sex differences in a unique pattern to that induced by P80 treatment.

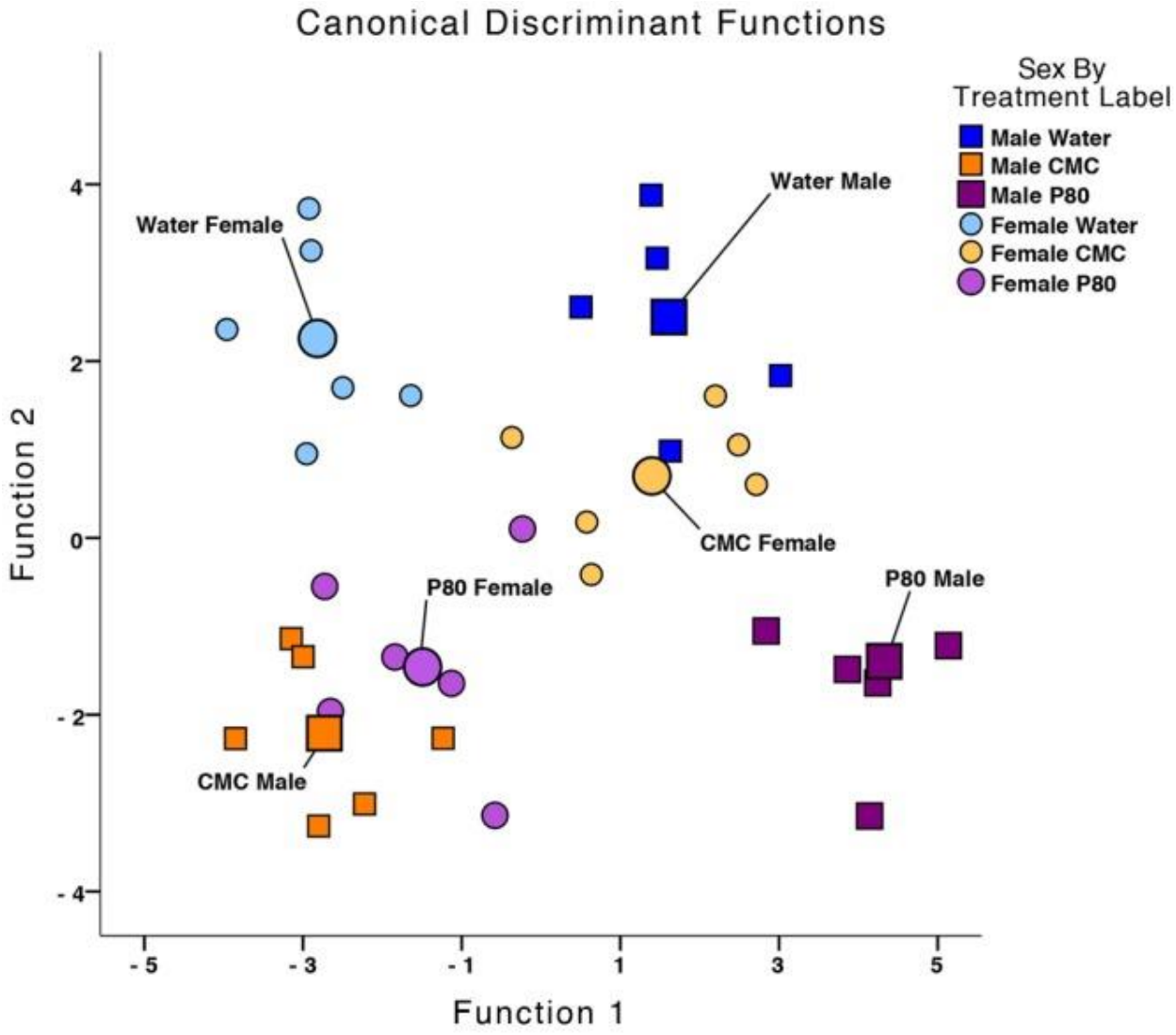

Figure 3.7 Dietary emulsifiers induce a syndrome of behavioral, physiological, and neural changes.

Multivariate analysis of the impact of emulsifiers on synergistic changes in behavioral measures, physiological parameters, and immunoreactivity of neuropeptides in the PVT.

The canonical discriminant function plots and Wilk's lambda revealed significant separation of groups by sex and emulsifier treatment $\Lambda = 0.003$, $X^2(138.194) = 73.182$, $p < 0.001$. *The group centroids for Function 1 are located in opposite directions with respect to the water-treated controls.*

Table 3.3 Functions at Group Centroids of Physiological, Behavioral, and Neuropeptidergic Effects of Emulsifier Treatment.

Sex by Treatment Label	Function				
	1	2	3	4	5
Water Male	1.601	2.495	-1.438	.858	-.714
CMC Male	-2.710	-2.215	-1.220	-.663	-.514
P80 Male	4.344	-1.401	-.619	-.209	.680
Water Female	-2.813	2.266	-.128	-.435	.845
CMC Female	1.374	.694	1.992	-.810	-.570
P80 Female	-1.528	-1.424	1.175	1.402	.155

Unstandardized canonical discriminant functions evaluated at group means.

3.5 Discussion

The increased incidence of disorders related to anxiety and anti-social behavior has led to the belief that substances to which humans have been exposed as a result of industrialization might impact brain function. Such substances do not need to have direct contact with the brain. Rather, substances that impact the gut-brain axis and/or

the intestinal microbiome might influence brain function and, consequently, behavior. In accord with this notion, we report here that the synthetic dietary emulsifiers CMC and P80, which have previously been shown to impact gut microbiota to induce low-grade inflammation and metabolic disorders, can also influence behavior. Specifically, we observed herein that consumption of CMC and P80 alters anxiety-like and sociability behavior. Such differences occurred in a sex-specific manner with distinct patterns of change in microbiota, neuropeptide expression, and behavior in male and female mice. Taken together, these data suggest that the sex-specific changes to microbiota composition induced by emulsifier consumption could drive the sex differences in physiological, neural, and behavioral effects of dietary emulsifiers.

Sex-specific patterns in brain and behavior were paralleled to some extent by differences in metabolism. Specifically, despite emulsifiers clearly promoting adiposity in male and female mice, an increase in overall weight was only seen in males (**Supplemental Figure 5**). This discrepancy may arise because we only weighed the perigonadal (periepididymal in males; periovarian in females) white adipose tissue. While mice have several other fat depots (Casteilla et al., 2008; White and Tchoukalova, 2014), the perigonadal adipose tissue pad has previously been demonstrated to respond to dietary changes (Rodriguez et al., 2004; Benz et al., 2012). When male and female mice are placed on calorie restriction following a high fat diet, females show a reduction predominantly in the gonadal fat pad size whereas males show a reduction in overall fat mass (Benz et al., 2012). Nonetheless, the differences observed between the effects of emulsifier treatment on adiposity and the relative body

weight in females suggest that emulsifiers induce a sex-specific change in body composition.

Sex-specific changes on spleen and colon weights following emulsifier consumption may result from sex-specific alterations of the composition of the gut microbiota following emulsifier treatment. The current study found sex differences in the microbiota of water-treated controls from both the previous study (Chassaing et al., 2015) and the present one, consistent with studies demonstrating sex differences in microbiota in C57Bl/6J mice (Org et al., 2016). Emulsifier treatment eliminated many of these sex differences, demonstrating the strong impact of emulsifiers on microbiota composition in yet another way. In addition, we observed sex-specific changes in microbiota composition of mice treated with emulsifiers, suggesting that some of the sex differences seen in the physiology and behavior may arise from the microbiota. While microbiota composition analysis and behavior assessment were performed after 84 and 49 days of emulsifier exposure, we previously reported that dietary emulsifier effects on the microbiota are rapid and seen *in vitro* after only few days of exposure (Chassaing et al., 2017).

Although emulsifier treatment induced changes in anxiety-like behavior, those changes cannot easily be interpreted in terms of changes in anxiety levels. For example, in males, emulsifier treatment reduced the time spent in the center portion of the open field without altering locomotive behavior or anxiety-like behaviors in the elevated plus maze, light/dark box, or marble burying test. This discrepancy between these three tests might mean that emulsifier exposure impacts passive coping or normal anxiety, for which the open field test has been suggested to be a more sensitive test

than for active coping or pathological anxiety (Prut and Belzung, 2003; Nosek et al., 2008). Moreover, the multivariate analysis showed that the aggregate of behaviors in the open field test (e.g, numbers of jumps, ambulatory episodes, circles, etc.) differed in emulsifier versus water-treated animals. In addition, emulsifier consumption increased the distance travelled in the elevated plus maze but not in the open field test. Although these effects cannot easily be interpreted in terms of changes in levels of anxiety or activity, they suggest that emulsifier treatment fundamentally impacts the organization of behavioral patterns.

Sex-specific alterations of the microbiota may have led to sex-specific changes in behavior, as found, for example, by the emulsifier-induced reduction in time spent in the center of the open field in males but not in females, or in social behavior in females but not male mice. A recent report indicates that offspring of dams fed on a high fat diet have social deficits, and that microbiota transplantation of such dysbiotic microbiota is sufficient to transfer such social deficits (Buffington et al., 2016). These data suggest that specific alterations of the gut microbiota may be critical for the behavioral effects of emulsifier treatment (Buffington et al., 2016). It is important to note that while our previous data using an *in vitro* microbiota system and fecal microbiota transplantation to germfree mice demonstrated that the detrimental effects of emulsifiers on metabolism are driven by direct effects on the intestinal microbiota (Chassaing et al., 2015; Chassaing et al., 2017), we cannot yet rule out that emulsifier-effects on brain and behavior are microbiota-independent.

Effects of emulsifier treatment on weight gain may be reflected in the increases of AgRP-IR in the arcuate nucleus, the location of the AgRP-expressing neuronal cell

bodies, and reductions in αMSH-IR in the PVT, an area that projects to key regions that contribute to both food intake and anxiety-like behaviors (reviewed in Kirouac, 2015; Vertes et al., 2015). AgRP stimulates food intake and reduces anxiety-like behaviors, while αMSH inhibits food intake and increases anxiety-like behaviors (Kokare et al.; Liu et al., 2007; Dietrich et al., 2015). Therefore, if changes in peptide levels directly correlate with changes in peptide release, the changes in AgRP-IR and αMSH-IR are consistent with the increase in food intake by emulsifiers noted in our original study (Chassaing et al., 2015). Given that we did not observe a general increase in anxiety-related behaviors across tests in the current study, the relationship of the changes in AgRP-IR and αMSH-IR with anxiety-related behaviors is unclear.

Emulsifier treatment effects on behavior were not reflected in effects on vasopressin or on the microglial population, which did not change significantly. It is important to note, however, that we only measured the microglial marker Iba1-IR, and therefore can not rule out that other neuroinflammatory markers, such as interleukin-6 (IL-6) or activation of the NFKB pathway, are increased following emulsifiers, as they increase following high-fat diet (Thaler et al., 2012).

While determining the extent to which studies in mice are relevant to humans is inherently difficult, even in studies of metabolism where human and mice can be assayed by quite similar assays, it is especially hard to do so for behavioral disorders, whose complexity and heterogeneity make them difficult to model in mice. This caveat notwithstanding, we submit that our data support the general notion that some cases of behavioral disorders may be impacted by exposure to modern chemical stressors and, more specifically, that synthetic dietary emulsifiers may be one such stressor. Given the

ability of behavior to impact metabolic disorders, for example by impacting food consumption or energy expenditure, it is very difficult to disassociate CMC and P80's effects on metabolism and behavior. Rather, we submit that such effects are likely intertwined, which may generally reflect the increased societal incidence of a broad range of diseases associated with inflammation. Our results thus indicate that dietary emulsifiers may be one specific perturbant of the gut-brain axis that can promote such diseases. Identification of the range of substances that can likewise perturb this axis and, subsequently, reducing exposure to such substances may be a means to staunch disease states characterized by altered behavior.

3.6 Supplemental Figures

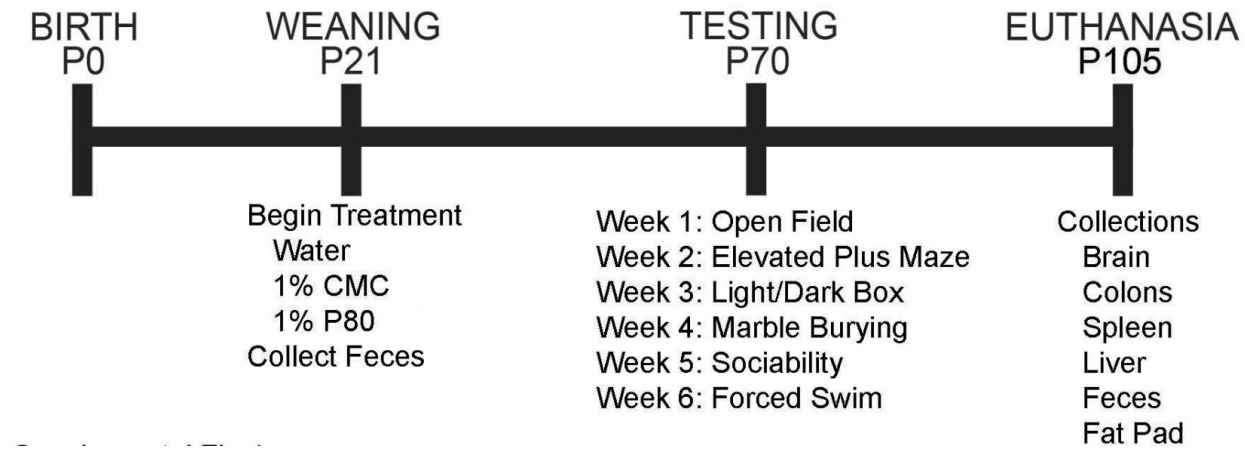

Figure 3.8 Supplemental Figure 1. Experimental Timeline.

Male and female C57Bl/6 mice were weaned on post-natal day 21 (P21), started on either water control or a 1% solution of either sodium carboxymethylcellulose (CMC) or polysorbate-80 (P80). In addition, feces were collected for microbiota analysis. Behavioral testing started at P70, with one test per week in the order indicated. One day

after completing the last behavioral test, animals were euthanized and feces, the brain and other organs were collected.

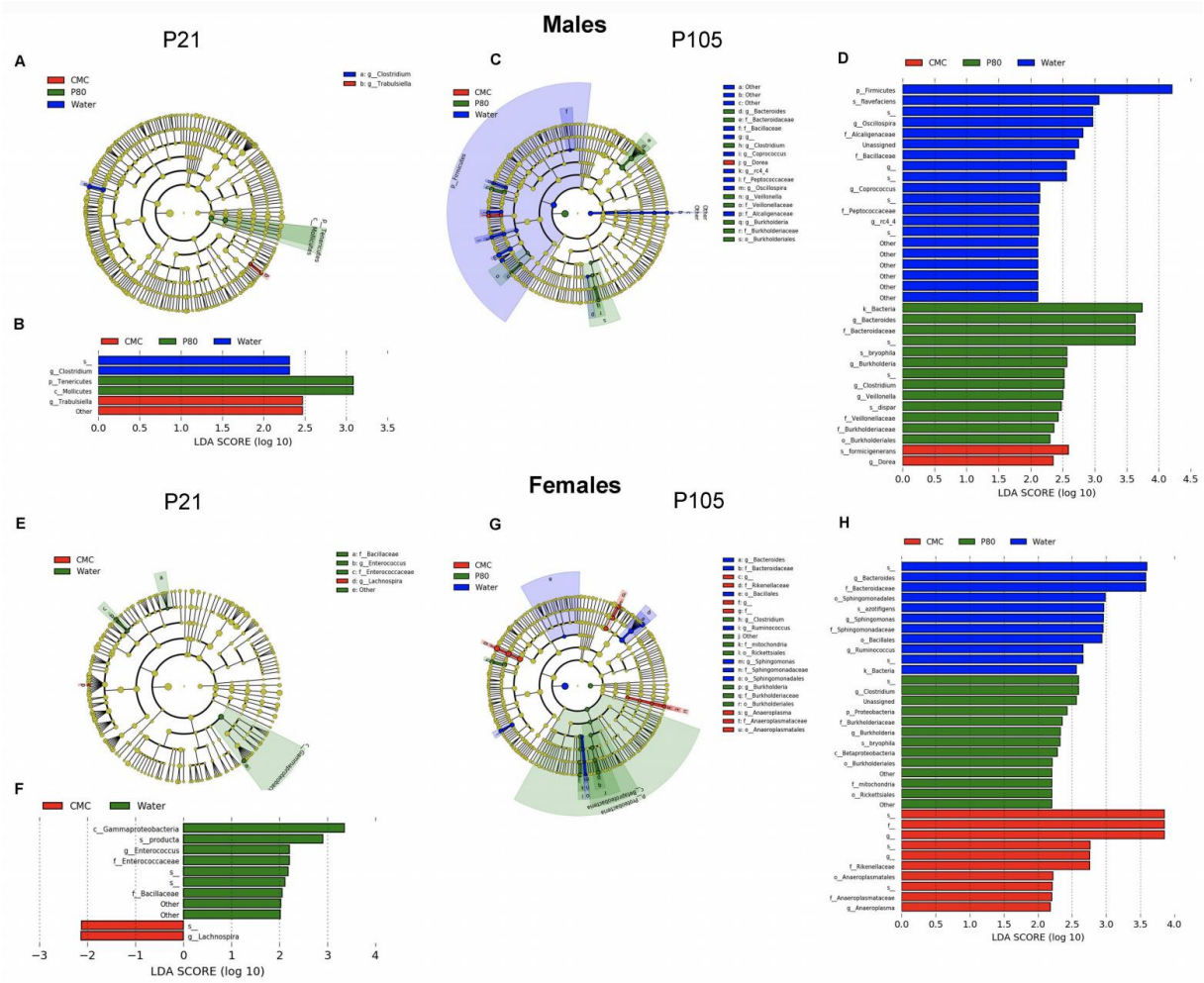

Figure 3.9 Supplemental Figure 2. Effects of dietary emulsifiers on microbiota.

Male and female C57Bl/6 mice were exposed to drinking water containing CMC or P80 (1%). Linear discriminant analysis coupled with Effect Size (LEfSe) was of taxa that differ significantly between male and female mice within water, CCM, and P80

treatments at time of weaning, P21 and at the time of collections, P105. Phylogenetic branching that differs by treatment within male (A, B) and female (E, F) mice at P21 and within male (C, D) and female (G, H) mice at P105.

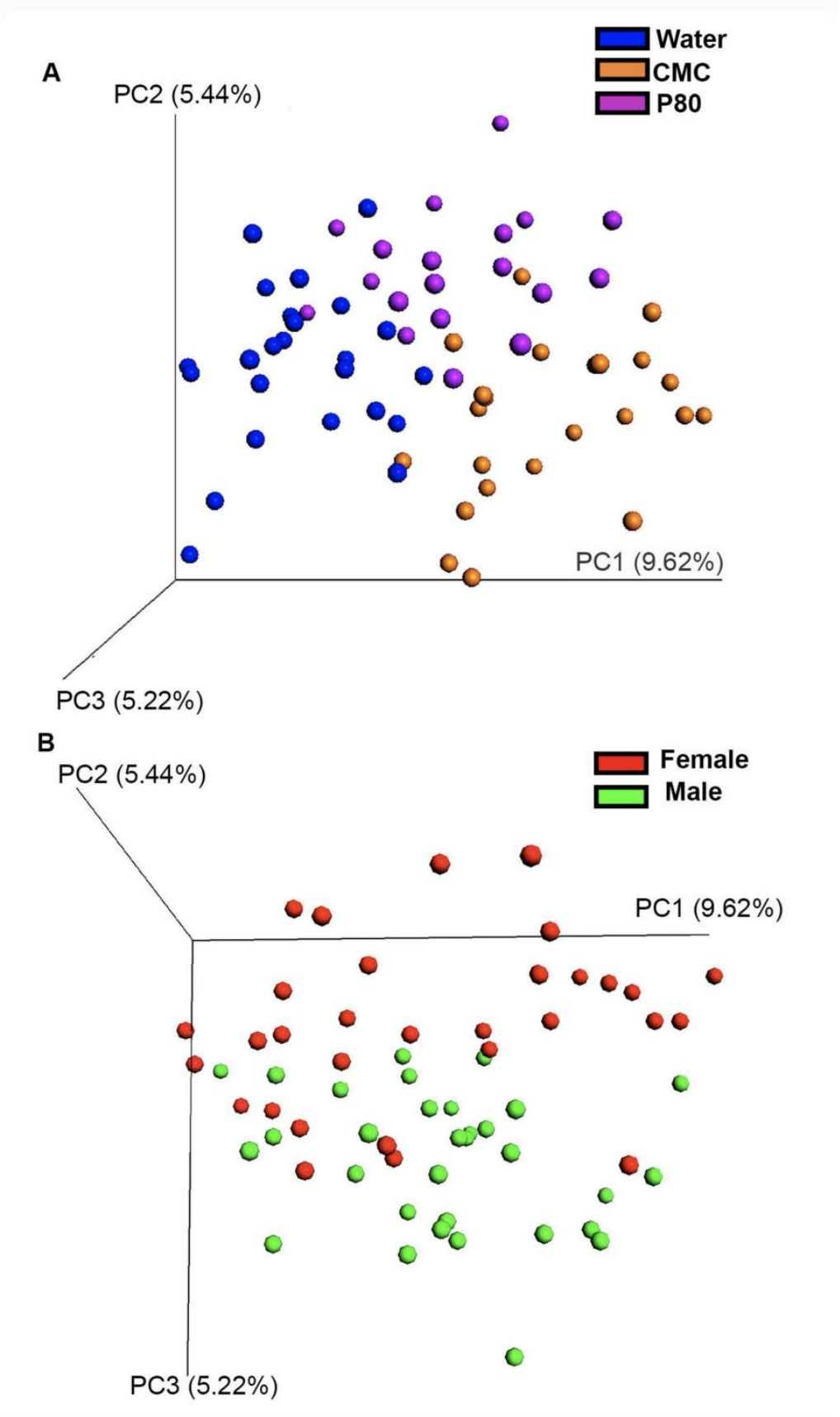
A
Water
CMC
P80
PC2 (5.44%)
PC1 (9.62%)
PC3 (5.22%)
B
Female
Male
PC2 (5.44%)
PC1 (9.62%)
PC3 (5.22%)

Figure 3.10 Supplemental Figure 3. Sex differences in microbiota in mice treated with dietary emulsifiers.

Male and female C57Bl/6 mice were exposed to drinking water containing CMC or P80 (1%) in data previously reported in Extended Data Figure 3 in Chassaing et al., 2015. Principal coordinates analysis (PCoA) of the unweighted UniFrac distance matrix of fecal microbiota showing clustering by treatment when male and female mice are combined into a single PCoA (A). Treatment group is indicated by point color (blue, water; orange, CMC; purple, P80). PCoA of the unweighted UniFrac distance matrix of fecale microbiota also show clustering by sex in (B). Sex is indicated by point color (red, female; green, male).

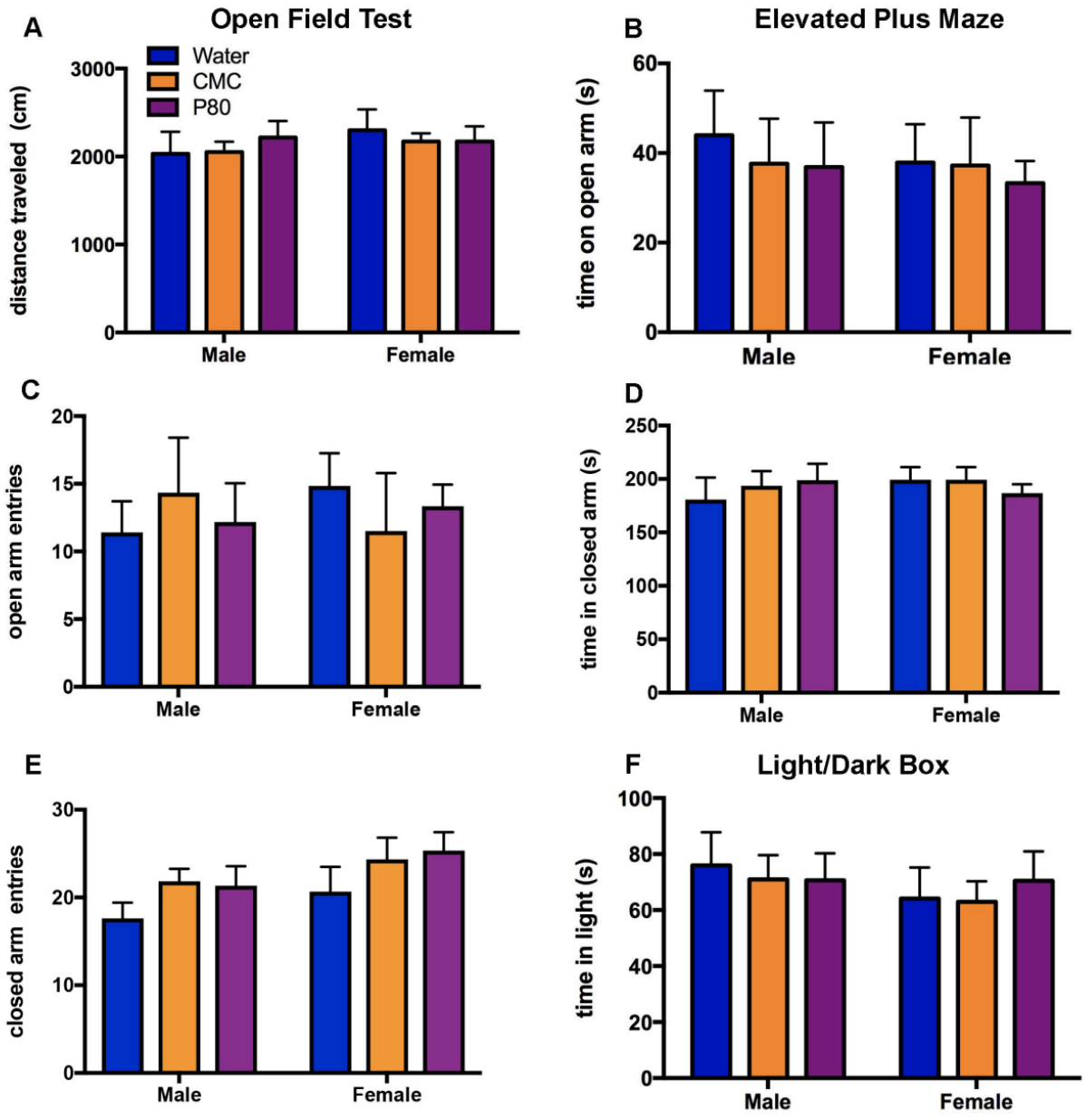

Figure 3.11 Supplemental Figure 4. Additional measures of anxiety-like behaviors in mice treated with emulsifiers.

Male and female C57Bl/6 mice were exposed to drinking water containing 3 CMC or P80 (1%) for 12 weeks and tested for anxiety-like behavior weekly starting at P70. (A) There was no effect of either emulsifier-treatment [F(2, 29) = 0.106, p=0.90] or sex [F(1, 29) = 0.59, p=0.45] on the distance traveled in the open field arena. (B) There was

no effect of either emulsifier-treatment [F(2, 29) = 0.1995, p=0.82] or sex [F(1, 29) = 0.1972, p=0.66] on the time spent on the open arms. (C) The number of entries onto the open arms was not affected by either emulsifier treatment [F(2, 29) = 0.006, p=0.99] or sex [F(1, 29) = 0.05, p=0.82]. (D) There was no effect of either emulsifier-treatment [F(2, 29) = 0.11, p=0.90] or sex [F(1, 29) = 0.13, p=0.73]on the time spent in the closed arms. (E) The number of entries into the closed arms was not affected by either emulsifier treatment [F(2, 29) = 2.18, p=0.13] or sex [F(1, 29) = 3.09, p=0.09]. (F) There was no effect of either emulsifier-treatment [F(2, 29) = 0.07, p=0.92] or sex [F(1, 29) = 0.68, p=0.41] on the time spent in the light in the light/dark box. Data are represented as means + SEM (n=5-6).

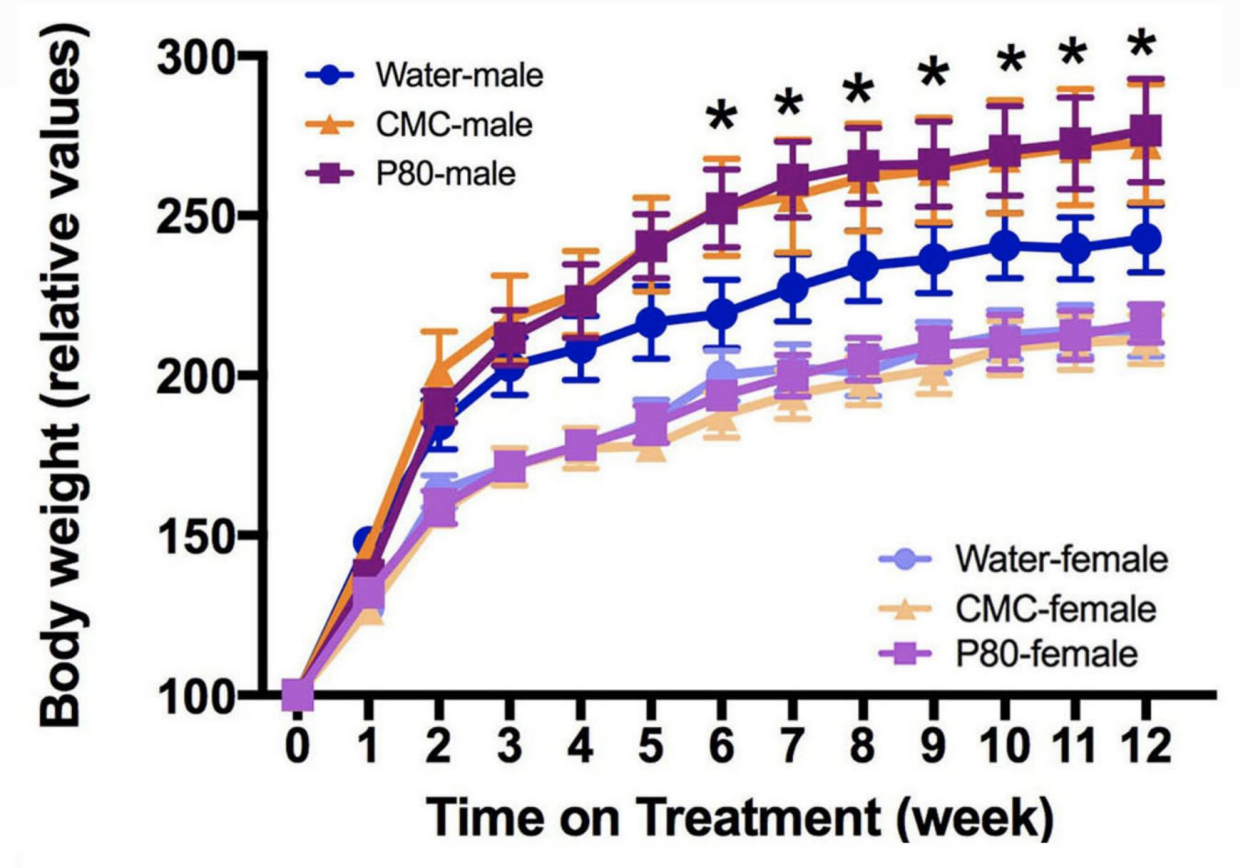

Figure 3.12 Supplemental Figure 5. Sex difference in relative weight gain in mice treated with dietary emulsifiers.

Male and female C57Bl/6 mice were exposed to drinking water containing CMC or P80 (1%) for 12 weeks. There was a significant interaction of time on treatment, treatment, and sex on the relative body weights in the mice over time [$F(24, 348) = 1.863$, $p<0.05$]. In addition, post-hoc analyses indicated that 6 weeks of emulsifier consumption lead to a greater body weight in male, but not female mice ($p<0.05$). Data are represented as means + SEM (n=5-6).*

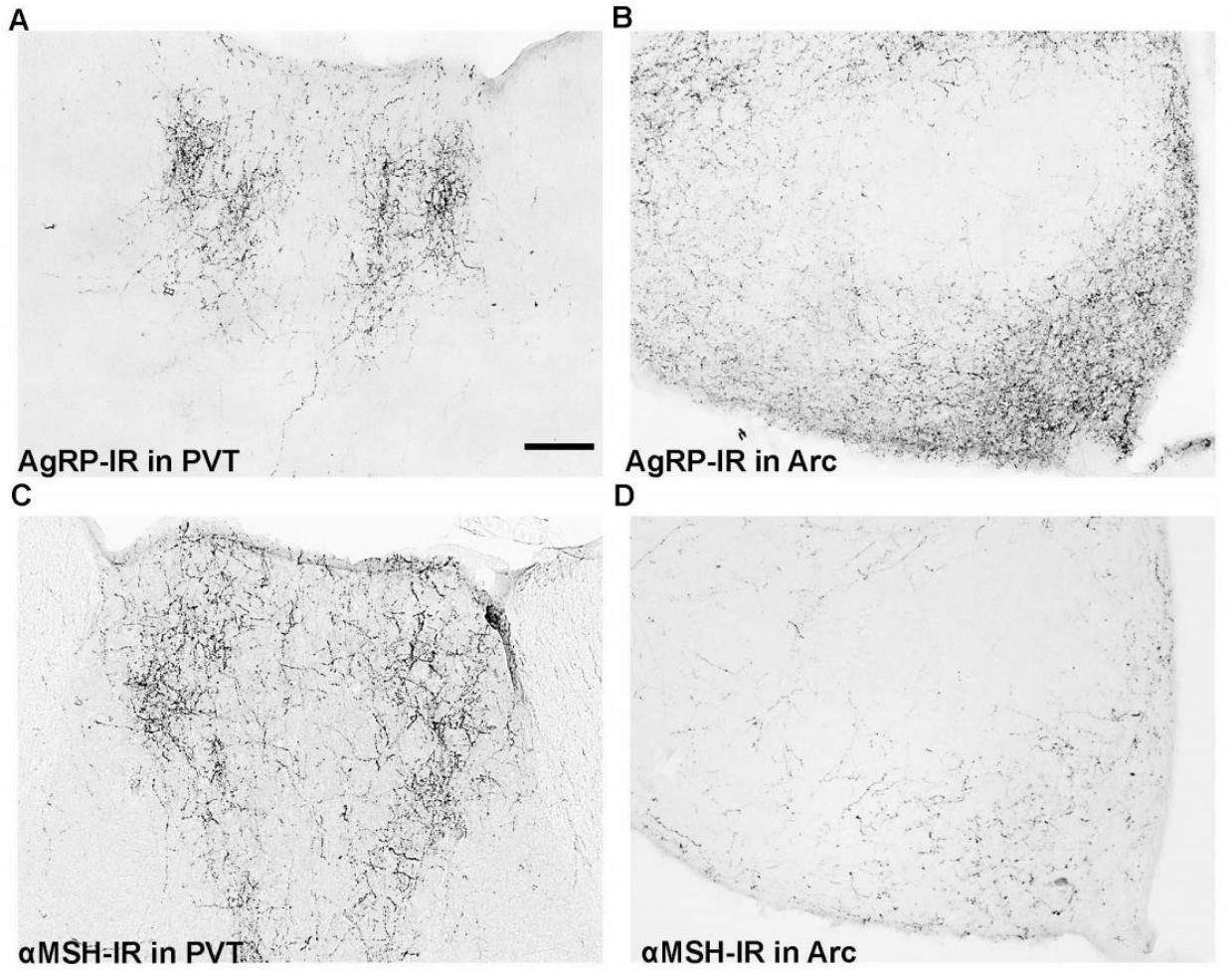

Figure 3.13 Supplemental Figure 6. Representative photomicrographs of Agouti-Related Peptide (AgRP) and alpha-melanocortin stimulation hormone (αMSH).

Photomicrographs showing the immunoreactivity (IR) for AgRP in (A) the paraventricular nucleus of the thalamus (PVT) and (B) the arcuate nucleus (Arc). Photomicrographs showing αMSH-IR in (C) PVT and (D) Arc. Scale bar: 100µm.

4 EFFECTS OF GUT-DERIVED ENDOTOXIN ON ANXIETY-LIKE AND REPETITIVE BEHAVIORS IN MALE AND FEMALE MICE

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4.1 Abstract

Gut dysbiosis is observed in several neuropsychiatric disorders exhibiting increases in anxiety behavior, and recent work suggests links between gut inflammation and such disorders. One source of this inflammation may be lipopolysaccharide (LPS), a toxic component of gram-negative bacteria. Here, we (1) determine whether oral gavage of LPS, as a model of gut-derived endotoxemia, affects anxiety-like and/or repetitive behaviors; (2) test whether these changes depend on TLR4 signaling; and (3) test the extent to which gut-derived endotoxin and TLR4 antagonism affects males and females differently.

In experiment 1, male wild-type (WT) and Tlr4-/- mice were tested for locomotor, anxiety-like, and repetitive behaviors in an automated open field test apparatus, 2 h after oral gavage of LPS or saline. In experiment 2, male and female WT mice received an oral gavage of LPS and an injection of one or two TLR4 antagonists that target different TLR4 signaling pathways ((+)-naloxone and LPS derived from *R. sphaeroides*

(LPS-RS)). Univariate and multivariate analyses were used to identify effects of treatment, sex, and genotype and their interaction.

In experiment 1, oral gavage of LPS increased anxiety-like behavior in male WT mice but not in Tlr4-/- mice. In experiment 2, oral gavage of LPS increased anxiety-like and decreased repetitive behaviors in WT mice of both sexes. Neither antagonist directly blocked the effects of orally administered LPS. However, treatment with (+)-naloxone, which blocks the TRIF pathway of TLR4, had opposing behavioral effects in males and females (independent of LPS treatment). We also identified sex differences in the expression of interleukin-6, a pro-inflammatory cytokine, in the gut both in basal conditions and in response to LPS.

In spite of the ubiquitous nature of LPS in the gut lumen, this is the first study to demonstrate that intestinally derived LPS can initiate behavioral aspects of the sickness response. While an increased enteric load of LPS increases anxiety-like behavior in both sexes, it likely does so via sex-specific mechanisms. Similarly, TLR4 signaling may promote baseline expression of repetitive behavior differently in males and females. This study lays the groundwork for future interrogations into connections between gut-derived endotoxin and behavioral pathology in males and females.

4.2 Introduction

Dysbiosis of the gut microbiota, defined as a shift toward pathological, pro-inflammatory microbial species, has been linked to a number of neuropsychiatric disorders associated with increased expression of anxiety behavior, including autism (Finegold et al., 2012; Mayer et al., 2014b; Mayer et al., 2014a), ADHD (Petra et al.,

2015), and psychological pathologies co-morbid with inflammatory bowel disease (IBD) (Bannaga and Selinger, 2015; Ray and Dittel, 2015). Microbiota-induced gut inflammation may mediate these behavioral pathologies. An important agent in these effects is likely to be lipopolysaccharide (LPS), a pathogenic component of gram-negative bacteria, which is endogenous to the gut microbiota (Marshall, 2005). When injected systemically, LPS produces well-documented behavioral alterations collectively called “sickness behavior,” which include an increase in anxiety-like behaviors (Dantzer, 2001, 2009) and suppression of compulsive and repetitive behaviors (Liblau et al., 1995; Gentile et al., 2016; Sung et al., 2016).

Intraperitoneal injections of LPS allow direct exposure of LPS to extra-intestinal peritoneal leukocytes that produce systemic cytokines that will provoke a sickness response. Likewise, intravenous injections of LPS facilitate its fast and robust interaction with splenic immune cells and circulating leukocytes. However, it is unknown whether elevations of serum LPS levels originating from gut barrier dysfunction, observed in rodent models of gut dysbiosis (such as emulsifier-fed mice (Chassaing et al., 2015), mice with dextran sodium sulfate (DSS)-induced colitis (Gabele et al., 2011; Chassaing et al., 2014b), high fat diet fed mice (Bruce-Keller et al., 2015), and toll-like receptor 2 knockout (*Tlr2-/-*) mice (Caricilli et al., 2011)), are responsible for increases in anxiety-like behavior observed in these models. These studies reliably demonstrate a 2- to 3-fold increase in serum LPS levels in experimental subjects relative to controls, a condition termed “metabolic endotoxemia” (Cani et al., 2007). As even a 10μg/kg dose of LPS (10 times lower than in most published studies) is sufficient to increase serum levels of LPS to 25X above baseline (Hansen et al., 2000), it is questionable whether

intraperitoneal injections recapitulate the dynamics of LPS-induced inflammation observed in “metabolic endotoxemia”. Furthermore, the site of action may make a difference, as an inflammatory stimulus injected intraperitoneally may differ in its neurobehavioral effects from an inflammatory stimulus administered orally.

Under most circumstances, LPS present on gut bacteria does not cause pathology. However, increased intestinal loads of LPS may breach the intestinal lining, activate intestinally-associated innate immune cells, and produce metabolic endotoxemia (Cani et al., 2007). Elevated gut levels of gram-negative bacteria have been reported in clinical populations, such as children with autism or individuals with celiac disease (Nadal et al., 2007; Finegold et al., 2010). Furthermore, the severity of gastrointestinal conditions correlates positively with levels of anxiety behavior in autistic children (Mazurek et al., 2013; Hsiao, 2014; Gracie et al., 2016; Reigada et al., 2016b). In addition, elevated fecal levels of LPS are reported for rodent models of diseases such as diet- and emulsifier-induced obesity, as well as colitis; microbiota transfer from each of these disease models into control subjects causes similar immune and/or behavioral deficits to those observed in the respective disease model (Bruce-Keller et al., 2015; Jang et al., 2017b; Lim and Kim, 2017; Viennois et al., 2017). However, whether gut-derived LPS influences anxiety behavior remains untested.

The purpose of this study is to: 1) determine whether oral gavage of LPS, as a model of gut dysbiosis, affects anxiety-like and/or repetitive behaviors, 2) test whether these changes depend on TLR4 signaling, and 3) test the extent to which gut-derived endotoxin and TLR4 antagonism affects males and females differently. Here we show that LPS triggers behavioral changes in males as well as females, but the underlying

signaling mechanisms may differ. Furthermore, the effects of gut-derived LPS may not depend on systemic TLR4 signaling.

4.3 Materials and Methods

4.3.1 Animals

Three-month old C57Bl/6J mice were used to test the effects of gut-derived LPS on anxiety-like and repetitive behaviors. For Experiment 1, 14 male wildtype (C57Bl/6J) mice and 8 male *Tlr4-/-* (*Tlr4*$^{lps\text{-}del}$ on C57Bl/6 background) mice were randomly selected from a colony bred in-house. Founder mice for this colony were sourced from Jackson Labs (Bar Harbor, ME). As this colony contained a negligible number of females, female subjects were not used in this experiment. For Experiment 2, 64 male and 64 female C57Bl/6J mice were purchased at 10 weeks of age (Jackson Labs) and housed in our facility for two weeks prior to study. For both experiments, all subjects were housed in same-sex pairs prior to the beginning of the study. The mice were housed in a room maintained in a 12:12 light dark cycle, at 68-72°F and approximately 50% humidity and were fed ProLab 5001 Diet *ad libitum* (LabDiet, St. Louis, MO). All experimental protocols were approved by the Institutional Animal Care and Use Committee at Georgia State University and were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All procedures were designed to minimize subject discomfort and use the fewest animals necessary for statistical analysis.

4.3.2 Experiment 1

On the day of testing, mice were single-housed and fasted for two hours to ensure gastric emptying (Firpo et al., 2005) and were then administered 300µg/kg LPS in a total volume of 200µl saline (LPS from *Escherichia coli* [O111:B4]; Sigma, St. Louis, MO) or saline alone by oral gavage (7 per treatment group for WT mice and 4 per treatment group for *Tlr4-/-* mice). The tip of the gavage needle was dipped in a 30% sucrose solution to decrease gavage-related stress response (Hoggatt et al., 2010). Two hours after gavage treatment, subjects were transferred to an automated open field apparatus for 10 minutes to measure locomotor parameters (ambulatory episodes, ambulatory counts, ambulatory time, ambulatory distance, resting time, and average velocity), anxiety-like behaviors (time spent in the center zone, zone entries, number of rears, and time spent rearing), and repetitive behaviors (time spent in stereotypic circling, number of stereotypic counts, jump counts, jump time, number of clockwise reversals, and number of counter-clockwise reversals). After behavior testing, serum samples were collected by terminal cardiac puncture blood collection under isoflurane anesthesia.

4.3.3 Experiment 2

To determine which TLR4 signaling cascade is responsible for the anxiogenic effects of orally administered LPS, we used (+)-naloxone (NIDA Drug Supply), which blocks the TLR4/TRIF cascade and has low affinity for mu-opioid receptors (1/1000 to 1/10000 the affinity of (-)-naloxone for mu-opioid receptors) (Iijima et al., 1978; Marcoli et al., 1989), and LPS-RS Ultrapure (InVivoGen, San Diego, CA) (LPS derived from

Rhodobacter sphaeroides, hereafter simply referred to as LPS-RS), which blocks the TLR4/MyD88 cascade(Li et al., 2014). LPS molecules, sourced from different bacterial species and strains, differ in level of immunogenicity, ranging from TLR4 agonists, such as *E. coli* derived LPS, that produce robust inflammation, to TLR4 antagonists, such as LPS derived from *R. sphaeroides* (LPS-RS), that block the inflammatory effects of pro-inflammatory LPS species (Coats et al., 2005; Vatanen et al., 2016). We selected a dose of 60mg/kg of (+)-naloxone, administered 30 minutes prior to LPS challenge, as applying this dose and timing blocks sedation and motor impairments induced by acute exposure to ethanol, a condition associated with increased intestinal permeability (Wu et al., 2012; Corrigan et al., 2015). We selected a dose of 800µg/kg of LPS-RS, injected 30 minutes prior to oral gavage of LPS, as intrathecal injection of this dose and timing has been demonstrated to block neuropathic pain induced by LPS (Sorge et al., 2011).

To ensure gastric emptying, male and female mice were single-housed and fasted for two hours (Firpo et al., 2005). Ultimately, 8 mice of each sex were assigned to each 2 (gavage treatment) x 2 ((+)-naloxone treatment) x 2 (LPS-RS treatment) group. Ninety minutes into the fast, mice received 60mg/kg (+)-naloxone in 200µl saline, 800 µg/kg LPS-RS in 200 µl saline, (+)-naloxone and LPS-RS together in 200µl saline, or 200µl saline by i.p. injection. Thirty minutes later, subjects received saline or 300µg/kg LPS by oral gavage. As in Experiment 1, the tip of the gavage needle was dipped in a 30% sucrose solution prior to insertion. Two hours after the oral gavage, mice were tested on an automated open field apparatus as above. Directly after behavior testing, mice were euthanized for serum and intestinal tissue collection.

4.3.4 Serum LPS

Hemolysis-free serum was generated by centrifugation of blood using serum separator tubes (Becton Dickerson, Franklin Lakes, NJ). Serum was stored at -20°C in silanized tubes. On the day of analysis, serum was diluted 1/40 in LPS-free saline, and residual plasma proteins were degraded via a 10 minute 70°C incubation (Caricilli et al., 2011). Serum LPS concentrations were determined using a kit based on a Limulus amebocyte extract (GenScript, Piscataway, NJ) according to manufacturer's instructions, with samples run in duplicate.

4.3.5 RT-qPCR for intestinal tissue

We examined the expression of pro-inflammatory (IL-1β, lL-6, TNF-α) and anti-inflammatory (IL-10) cytokines in the gut in response to oral LPS exposure. To do so, one inch of jejunum tissue was homogenized in trizol (Invitrogen, Carlsbad, CA) for RNA extraction. Reverse transcription was performed with a SuperScript IV First-Stand Synthesis Kit (Invitrogen) in a thermal cycler (Applied Biosystems Inc., Foster City, CA) and real time PCR was performed in the LightCycler 96 System (Roche, Mannheim, Germany) using FastStart Essential DNA Green Master Kit (Roche) according to the manufacturer's instructions and as previously described (Castillo-Ruiz et al., 2017). Primers used targeted messenger RNA for *IL-10, IL-1β, TNF-α, IL-6, and glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) as reference gene (all validated primers from Qiagen Inc., Valencia, CA).

4.3.6 Luminex cytokine assay

BioRad (Hercules, CA) 4-plex mouse Luminex kits were used to measure serum cytokine levels. The cytokines assayed were TNF-α, IL-1β, IL-6, and IL-10. Assays were performed according to the manufacturer's instructions, and samples were run in duplicate.

4.3.7 Statistical Analyses

Using SPSS (version 23), Univariate (ANOVA) and Multivariate Analysis of Variance (MANOVA) were performed on the data obtained from the automated open field test apparatus. Following ANOVAs, planned contrasts were performed on individual outcome variables to identify directionality between group differences. Multivariate statistics are useful to detect relationships between outcome variables and identify syndromes of behavioral effects, particularly in outcomes with statistically non-significant univariate ANOVAs (Cooley, 1971). Like ANOVA, which tests whether mean differences between groups on a single dependent variable occurs by chance, MANOVA tests whether mean differences for a combination of dependent variables occur by chance. Discriminant analysis ranks outcome variables by their contribution to group separation along the combination of all dependent behavioral variables used in the ANOVA analyses. The same group of behavioral measures were used in all discriminant analyses across Experiments 1 and 2. Only behavioral measures that differentiate the discriminant functions are listed in the structure matrix. Discriminant functions were validated with both original group case classification tests and leave-

one-out cross-validation tests, which gives a more unbiased estimate of the generalizability of the discriminant functions (Bishop, 1995; Ghani, 2009).

Effect sizes for sex, genotype, and treatment effects were reported as sample means with 95% confidence intervals. In addition, using SPSS, partial eta squared ("*partial* η^2") were reported as effect size measurements of variance within the ANOVA and MANOVA tests. Estimation of population means and 95% confidence intervals were calculated for WT males across Experiments 1 and 2 using random effects meta-analysis. All confidence interval estimates were calculated with Exploratory Software for Confidence Intervals or ESCI (Wolfe and Cumming, 2004; Perezgonzalez, 2015).

Two-way ANOVAs (sex by treatment) were performed for the analysis of gut cytokine expression using GraphPad Prism version 6.01. Significant effects were followed by Fisher's LSD tests.

4.4 Results

4.4.1 Experiment 1: Role of TLR4 in behavioral response to oral gavage of LPS

In line with other models of metabolic endotoxemia (Cani et al., 2007; Caricilli et al., 2011), oral gavage of LPS in male WT mice increased serum levels of LPS 1.5-fold, two hours after gavage treatment, $t(8)=16.96$, $p<0.05$ (one-tailed), n=5/group, $\eta^2=0.34$ (Cani et al., 2007; Caricilli et al., 2011) (**Fig. 4.1**).

2 x 2 (genotype x gavage treatment) univariate ANOVAs across all behavioral measures showed that oral gavage of LPS significantly increased anxiety-like behavior in WT mice, but not *Tlr4-/-* mice (n=7/group for WT mice and n=4/group for *Tlr4-/-* mice). In comparison to vehicle treatment, LPS decreased time spent in the center for WT

mice, $p<0.01$, but not in *Tlr4-/-* mice, (genotype x gavage treatment interaction: $F(1,18)=14.051$, $p<0.01$). If anything, LPS tended to increase time spent in the center for *Tlr4-/-* mice, although this did not reach significance, $p=0.107$ (**Fig. 4.2a**). When collapsing across gavage treatment, WT mice had a higher jump time, $p<0.001$ (**Fig. 4.2e**), whereas *Tlr4-/-* mice had a higher average velocity, $p<0.01$ (**Fig. 4.2b**), spent more time in stereotypic circling, $p<0.001$ (**Fig. 4.2c**), and had a higher number of stereotypic counts, $p<0.001$ (**Fig. 4.2d**). **Supplemental Table 1** lists ANOVA statistics for all measures across main effects and interactions, including locomotor parameters (ambulatory episodes, ambulatory counts, ambulatory time, ambulatory distance, resting time, and average velocity), anxiety-like behaviors (time spent in the center zone, zone entries, number of rears, and time spent rearing), and repetitive behaviors (time spent in stereotypic circling, number of stereotypic counts, jump counts, jump time, number of clockwise reversals, and number of counter-clockwise reversals).

A 2 x 2 (genotype x gavage treatment) Multivariate Analysis of Variance (MANOVA) was performed on all behavioral measures, wherein all main effects and interactions were non-significant (not reported). The observed power for the main effect of gavage treatment (power=20.9%), the main effect of genotype (power=46.1%), and the gavage treatment by genotype interaction effect (power=43.2%) were all under the nominal 80% level. This suggests that this experimental cohort may have been underpowered for a factorial MANOVA.

Although MANOVA didn't reveal significant effects of genotype or gavage treatment, discriminant analysis revealed the contribution of behavioral outcome variables to group separation by genotype and gavage treatment. When subjects were

designated to four groups based on genotype and gavage treatment, discriminant analysis revealed three discriminant functions that maximize group separation based on genotype, gavage treatment, or the interaction between these two factors. Function 1 explains 60.0% of the variance, canonical R^2=0.975, Function 2 explains 35.6% of the variance, canonical R^2=0.959, and Function 3 explains 4.4% of the variance, canonical R^2=0.765. Collectively, these discriminant functions significantly differentiated the treatment groups, Λ=0.006, $X^2(42)=77.077$, $p<0.001$. The structure matrix in Table 1 reveals the correlations between outcome variables and the discriminant functions. In the discriminant function plot (**Fig. 4.3**), Function 1 demonstrates the opposing effects of gavage treatment on behavioral outcomes via the two genotypes, and the structure matrix shows this is predominantly driven by time spent in center zone (r=-0.154), jump time (r=-0.138), and jump counts (r=-0.070) (**Table 4.1**). Function 2 separates groups by genotype, and this is driven predominantly by time spent in stereotypic circling (r=-0.458), number of stereotypic counts (r=-0.454), and time spent in the center zone (r=-0.279) (**Table 4.1**). This suggests that while the genotypes are best distinguished on the basis of repetitive behaviors, gavage treatment affects anxiety-like and repetitive behavior differently in male WT and *Tlr4-/-* mice. **Supplemental Table 2** demonstrates the results of a classification test to verify the validity of the discriminant functions plotted in **Figure 4.3**. Using the original discriminant functions, 100% of the original grouped cases are correctly classified. These functions were further validated by a leave-one-out cross-validation procedure. Discriminant functions were re-computed with all subjects excluding one and this procedure was repeated for all subjects. Across all

analyses, 63.6% of cross-validated group cases were correctly classified, 38.6% above chance.

Table 4.1 Experiment 1 Structure Matrix (Genotype by Gavage Treatment).

	Function		
Measured Outcome	1	2	3
Jump Counts	**-0.070**	0.070	-0.026
Number of Stereotypic Counts	0.003	**-0.458**	0.186
Time Spent in Stereotypic Circling	0.000	**-0.454**	**0.231**
Time Spent Jumping	**-0.138**	0.300	-0.129
Ambulatory Time	0.038	0.102	-0.028
Time Spent Rearing	-0.015	-0.099	-0.020
Number of Ambulatory Counts	0.025	0.069	0.045
Ambulatory Distance	0.032	0.044	0.024
Time Spent Resting	-0.017	-0.024	-0.014
Average Velocity	-0.031	-0.216	**0.390**
Time Spent in Center Zone	**-0.154**	**-0.279**	**0.333**
Number of Clockwise Reversals	-0.029	0.019	0.171
Number of Zone Entries	-0.020	-0.098	0.153
Ambulatory Episodes	0.019	-0.025	0.120
Number of Counter-Clockwise Reversals	-0.017	0.069	-0.075

Function numbers match the order of the percentage of the variance explained by the respective functions. Each function maximizes separation between groups based on main effect of genotype, main effect of gavage treatment, or an interaction between these two factors, on the listed behaviors. Boldfaced numbers indicated the highest three correlations, and therefore deemed most important for the discriminant function.

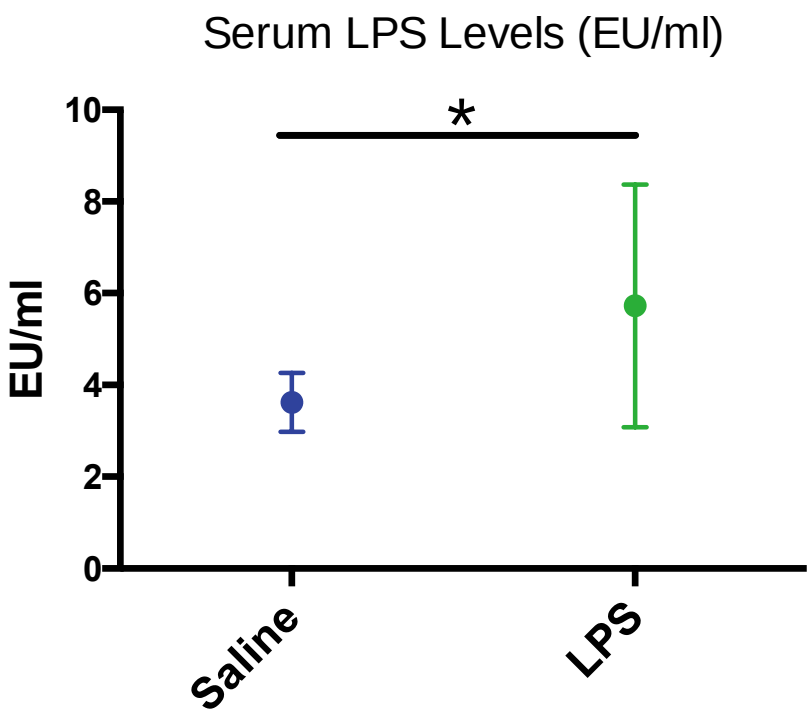

Figure 4.1 Oral gavage of LPS produces low-grade endotoxemia.

*Serum LPS levels in WT mice treated with saline (blue bar) or LPS (green bar). LPS treatment significantly increased LPS levels 1.5-fold, 2 h after gavage. Bars indicate mean and 95% confidence intervals. *p < 0.05*

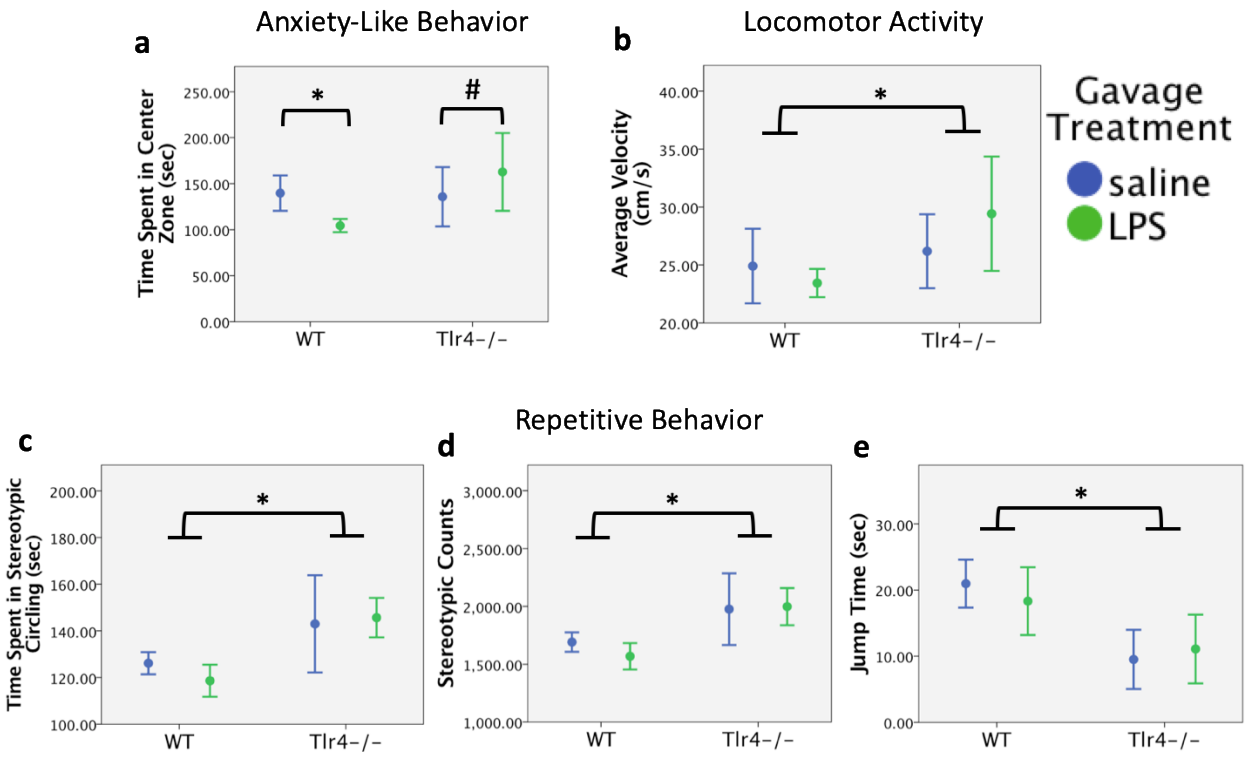

Figure 4.2 Effects of oral gavage of LPS on male WT and *Tlr4−/−* mice.

Experiment 1: effects of oral gavage of LPS on open field test behavioral outcomes in male WT and Tlr4−/− mice. ***a*** *LPS significantly decreased time in the center zone for male WT mice, but there was a slight trend toward increased time spent in the center zone for male Tlr4−/− mice.* ***b*** *Male Tlr4−/− mice had significantly increased average velocity,* ***c****time spent in stereotypic circling, and* ***d*** *number of stereotypic counts compared with male WT mice.* ***e*** *However, male WT mice had a significantly higher jump time compared to male Tlr4−/− mice. Error bars are 95% confidence intervals. *p < 0.05; #p = 0.107*

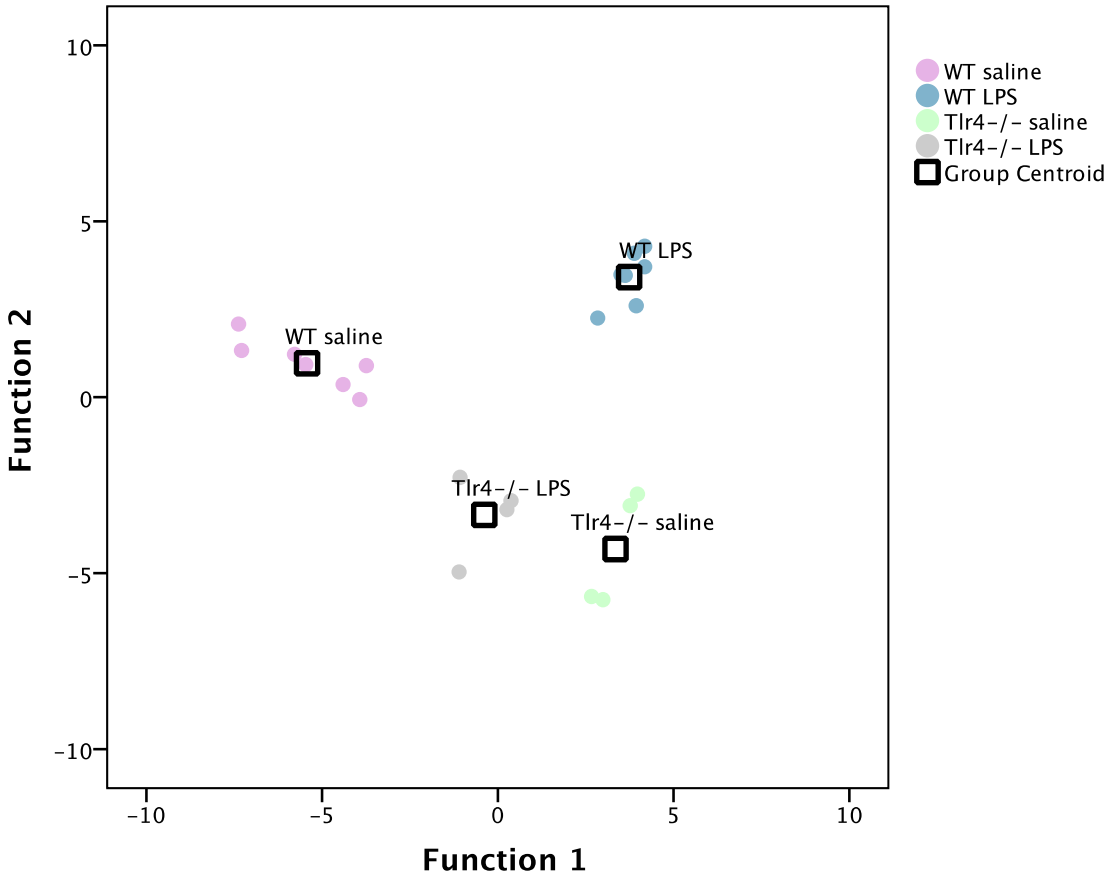

Figure 4.3 Canonical discriminant function plot for experiment 1.

Experiment 1 discriminant function plot. Correlations between outcome variables and discriminant functions are listed in Table 1. Function 1 demonstrates an interaction between gavage treatment and genotype, mostly based on the differential effect of LPS on time spent in the center zone, time spent jumping, and jump counts in Tlr4−/− *and WT mice, whereas function 2 separates groups based on genotype, largely driven by differences in number of stereotypic counts, time spent in stereotypic circling, and time spent in the center zone. Group centroids indicate the mean discriminant function value of each of the designated groups.*

4.4.2 Experiment 2: Sex differences in TLR4 agonism and antagonism on anxiety-like and repetitive behavior.

In this experiment, we observed that oral gavage of LPS significantly increased anxiety behavior in both males and females, but a specific TLR4 antagonist (+)-naloxone, had opposing effects on anxiety and repetitive behavior in males and females. Full factorial 2 (sex) x 2 (gavage treatment: LPS or saline) x 2 (i.p. injection: LPS-RS or saline) x 2 (i.p. injection: (+)-naloxone or saline) MANOVA was performed on the same dependent variables analyzed in Experiment 1 (n=8/group). There were significant main effects of sex ($F(16,96)=9.751$, $p<0.001$) and gavage treatment ($F(16,96)=2.111$, $p<0.05$), and a sex by (+)-naloxone interaction effect ($F(16,96)=2.176$, $p<0.05$). All remaining main effects and interactions, including effects of LPS-RS treatment, were non-significant (not reported). ANOVAs revealed these main effects and interactions across a number of behavioral parameters (**Supplemental Table 3**), of which subsequent planned contrasts indicated directionality.

These planned contrasts revealed that LPS suppressed repetitive and increased anxiety-like behaviors in males and females. Since no significant sex by LPS treatment effects were found in any of the ANOVAs or MANOVA, males and females were grouped together for subsequent analyses. LPS treatment decreased number of zone entries, $p<0.05$ (**Fig. 4.4a**), and increased number of rears, $p<0.05$ (**Fig. 4.4b**). Across both sexes, LPS treatment decreased time spent in stereotypic circling, $p=0.05$ (**Fig. 4.4c**), stereotypic counts, $p<0.05$ (**Fig. 4.4d**), and jump counts, $p<0.05$ (**Fig. 4.4e**).

When planned contrasts were run for the effects of (+)-naloxone in each sex separately, time spent in stereotypic circling (**Fig. 4.5a**), stereotypic counts (**Fig. 4.5b**),

and jump counts (**Fig. 4.5d**) were significantly affected in females ($p<0.05$, in each case) but not in males ($p>0.1$ in each case).

Discriminant analysis confirms that LPS increased anxiety behavior similarly in males and females, while (+)-naloxone had different effects in males and females. As for gavage treatment, discriminant analysis revealed three discriminant functions on data from subjects grouped by sex and gavage treatment. Function 1 explains 80.6% of the variance, canonical R^2=0.758, Function 2 explains 13.8% of the variance, canonical R^2=0.433, and Function 3 explains 5.6% of the variance, canonical R^2=0.292. Collectively, these discriminant functions significantly differentiated the treatment groups, Λ=0.317, $X^2(45)$=133.937, p<0.001. The discriminant function plot (**Fig. 4.6**) shows that Function 1 separates groups based on sex, and the structure matrix (**Table 4.2**) reveals this is predominantly driven by sex differences in jump counts (r=-0.616), jump time (r=-0.508), and ambulatory time (r=-0.392). Function 2 separates groups based on gavage treatment, and LPS appears to affect males and females in a similar fashion along outcome variables, predominantly number of rears (r=0.453), zone entries (r=-0.429), and stereotypic counts (r=-0.400). **Supplemental Table 4** displays results of the original grouping and leave-one-out classification tests. 64.6% of original grouped cases were correctly classified, and 45.7% of cross-validated grouped cases were correctly classified, 20.7% above chance. (The same group of behavioral measures used in Experiment 1 were also used in discriminant analyses performed for Experiment 2. Only behavioral measures that differentiate the discriminant functions are listed in the structure matrix.)

Discriminant analysis also indicates that (+)-naloxone affected repetitive and locomotor behaviors differently in males and females, revealing three discriminant functions (**Fig. 4.7**, **Table 4.3**). Rear count is automatically excluded from the discriminant analysis, based on its lack of contribution to the discriminant functions. Function 1 explains 81.5% of the variance, canonical R^2=0.764, Function 2 explains 11.8% of the variance, canonical R^2=0.410, and Function 3 explains 6.7% of the variance, canonical R^2=0.322. Collectively, these discriminant functions significantly differentiated the treatment groups, Λ=0.311, $X^2(45)$=136.022, p<0.001. As shown in the discriminant analysis where subjects are grouped by sex and gavage treatment, the discriminant function plot shows that function 1 separates groups based on sex and the structure matrix reveals this is predominantly driven by sex differences in jump counts (r=-0.623), jump time (r=-0.496), and ambulatory time (r=-0.416). Function 2 separates groups based on (+)-naloxone treatment, and (+)-naloxone appears to affect males and females differently along outcome variables, predominantly stereotypic counts (r=0.545), ambulatory episodes (r=0.526), and time spent in stereotypic circling (r=0.523). **Supplemental Table 5** displays results of the original grouping and leave-one-out classification tests. 65.4% of original grouped cases were correctly classified, and 48.0% of cross-validated grouped cases were correctly classified, 23.0% above chance.

Since neither TLR4 antagonist blocked the specific effects of LPS, we sought to identify the systemic and intestinal inflammatory effects of the oral LPS treatment in WT mice not treated with TLR4 antagonists. Gavage treatment resulted in non-significant elevation of serum endotoxin levels in male subjects in a meta-analysis across

Experiments 1 and 2 (p>0.05) (**Supplemental Fig. 1**), and there were no significant main effects of sex or gavage treatment, or interactions between sex and gavage treatment, on serum LPS levels in Experiment 2 (data not shown). In addition, cytokine Luminex was performed on serum samples from Experiment 2. There were no significant effects of gavage treatment on serum levels of TNF-α, IL-1β, IL-6, or IL-10 (data not shown). However, oral gavage of LPS did modulate IL-6 expression levels in intestinal tissue in a sexually differentiated manner (sex-by-treatment interaction: $F(1,21)= 12.38$, $p= 0.002$), increasing IL-6 expression in females ($p= 0.04$) while decreasing it in males ($p= 0.009$; **Fig. 4.8a**). IL-10, TNF-α, and IL-1β expression levels were not significantly altered by the oral LPS treatment (**Fig 4.8b-d**).

Table 4.2 Experiment 2 Structure Matrix (Sex by Gavage Treatment).

	Function		
Measured Outcome	1	2	3
Jump Counts	**-0.616**	-0.354	0.124
Jump Time	**-0.508**	-0.270	0.139
Ambulatory Time	**-0.392**	-0.193	-0.108
Ambulatory Counts	-0.368	-0.283	-0.116
Ambulatory Distance	-0.345	-0.270	-0.060
Resting Time	0.344	0.295	0.014
Ambulatory Episodes	-0.330	-0.204	-0.120
Average Velocity	-0.171	-0.161	0.160
Number of Rears	0.018	**0.453**	**0.276**
Zone Entries	0.057	**-0.429**	**0.338**
Stereotypic Counts	-0.028	**-0.400**	0.189
Time Spent in Stereotypic Circling	0.086	-0.372	0.154
Time Spent Rearing	0.144	0.285	0.223

Counter Clockwise Reversals	-0.114	-0.252	-0.055
Time Spent in Center Zone	0.024	-0.094	**0.463**
Clockwise Reversals	-0.042	-0.080	-0.081

Function numbers match the order of the percentage of the variance explained by the respective functions. Each function maximizes separation between groups based on main effect of sex, main effect of gavage treatment, or an interaction between these two factors, on the listed behaviors. Boldfaced numbers indicated the highest three correlations, and therefore deemed most important for the discriminant function.

Table 4.3 Experiment 2 Structure Matrix (Sex by (+)-Naloxone Treatment)

	Function		
Measured Outcome	1	2	3
Jump Counts	**-0.623**	0.319	-0.057
Jump Time	**-0.496**	0.123	0.122
Ambulatory Time	**-0.416**	0.414	-0.134
Counter Clockwise Reversals	-0.106	0.086	0.090
Stereotypic Counts	-0.046	**0.545**	-0.008
Ambulatory Episodes	-0.363	**0.526**	-0.140
Time Spent in Stereotypic Circling	0.070	**0.523**	0.044
Time Spent Resting	0.367	-0.485	0.068
Ambulatory Counts	-0.390	0.442	-0.166
Ambulatory Distance	-0.369	0.439	-0.188
Average Velocity	-0.180	0.397	**0.345**
Number of Rears	0.020	-0.285	0.174
Time Spent Rearing	0.144	-0.086	**0.327**
Clockwise Reversals	-0.042	0.181	**0.197**
Zone Entries	0.058	0.075	-0.138
Time Spent in Center Zone	0.022	-0.061	-0.104

Function numbers match the order of the percentage of the variance explained by the respective functions. Each function maximizes separation between groups based on main effect of sex, main effect of (+)-naloxone treatment, or an interaction between

these two factors, on the listed behaviors. Boldfaced numbers indicated the highest three correlations, and therefore deemed most important for the discriminant function.

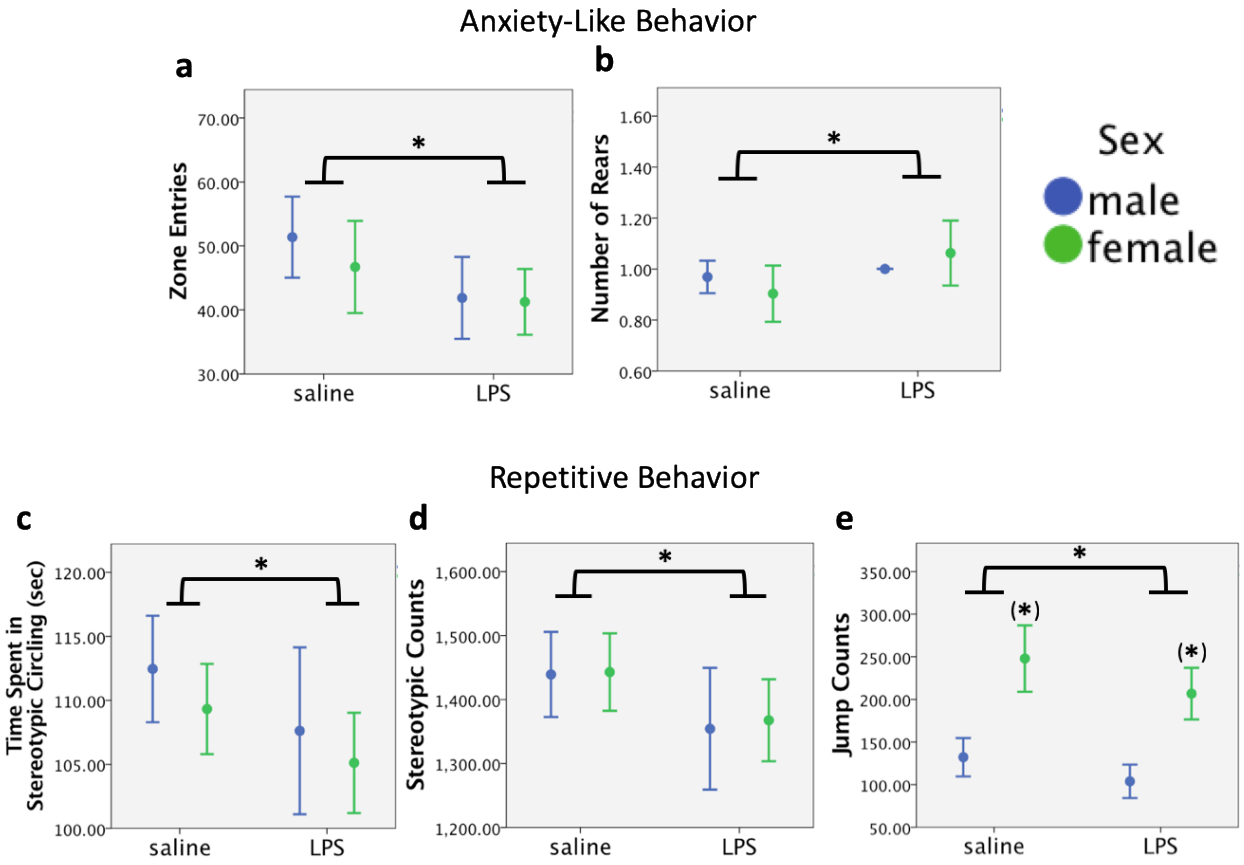

Figure 4.4 Effects of oral gavage of LPS on male and female WT mice.

*Experiment 2: effects of intraperitoneal injection of TLR4 antagonists and oral gavage of LPS on open field test behavioral outcomes in male and female WT mice. Data presented as sex by gavage treatment. LPS significantly **a** decreased zone entries and **b** increased the number of rears in males and females relative to saline-treated subjects. Furthermore, LPS significantly decreased the number of **c** time spent in stereotypic circling, **d** stereotypic counts, and **e** jump counts relative to saline-treated subjects. In addition, females had a higher jump count than males. Error bars are 95% confidence intervals. *p < 0.05; (*) significant main effect of sex, p < 0.05*

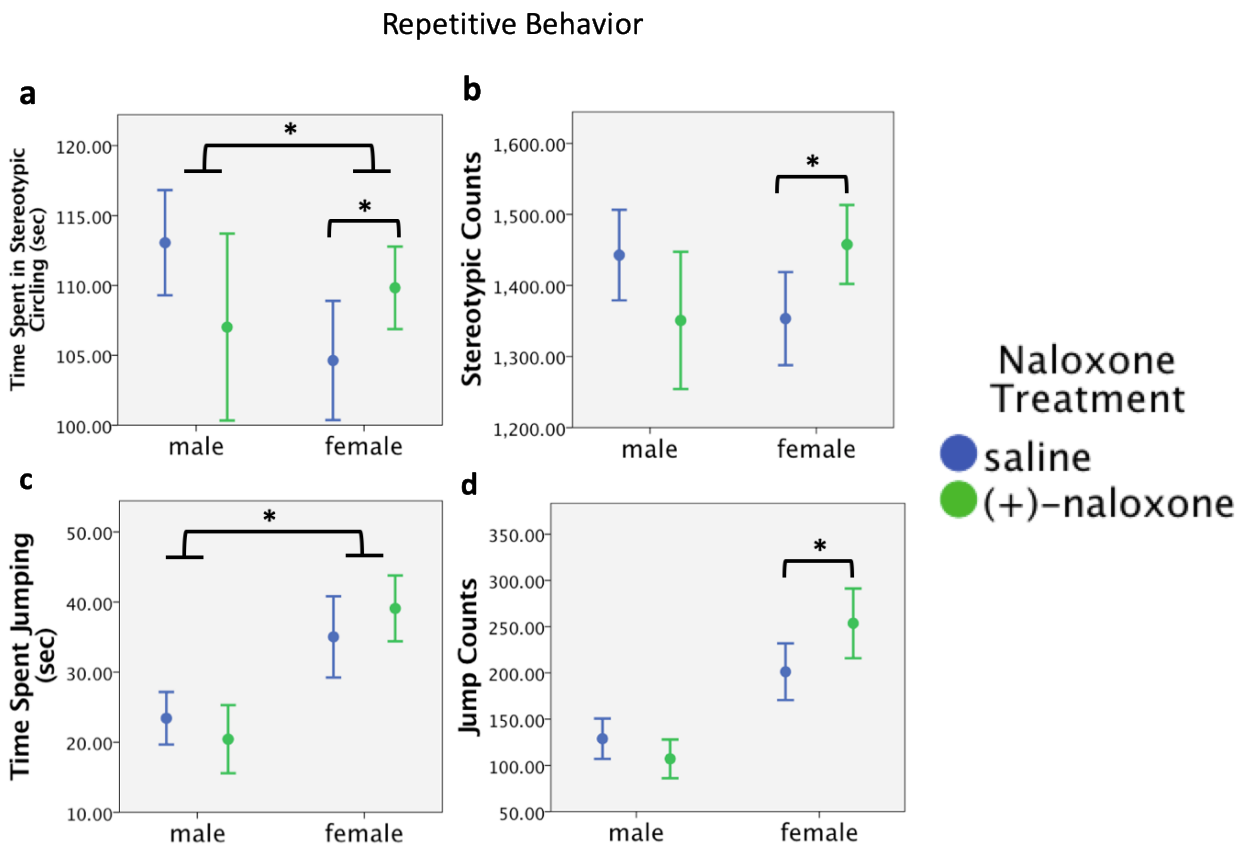

Figure 4.5 Effects of TLR4 antagonist (+)-naloxone treatment on male and female WT mice.

Experiment 2: effects of intraperitoneal injection of TLR4 antagonists and oral gavage of LPS on open field test behavioral outcomes in male and female WT mice. Data presented as sex by (+)-naloxone treatment. ***a****Overall, males spent significantly more time in stereotypic circling than females; however, (+)-naloxone significantly increased time in stereotypic circling in female mice.* ***b*** *In addition, (+)-naloxone significantly increased the number of stereotypic counts in female mice. For jumping behavior, (****c****) females jumped more than males, and* ***d*** *(+)-naloxone significantly increased jump counts in females. Error bars are 95% confidence intervals. *p < 0.05*

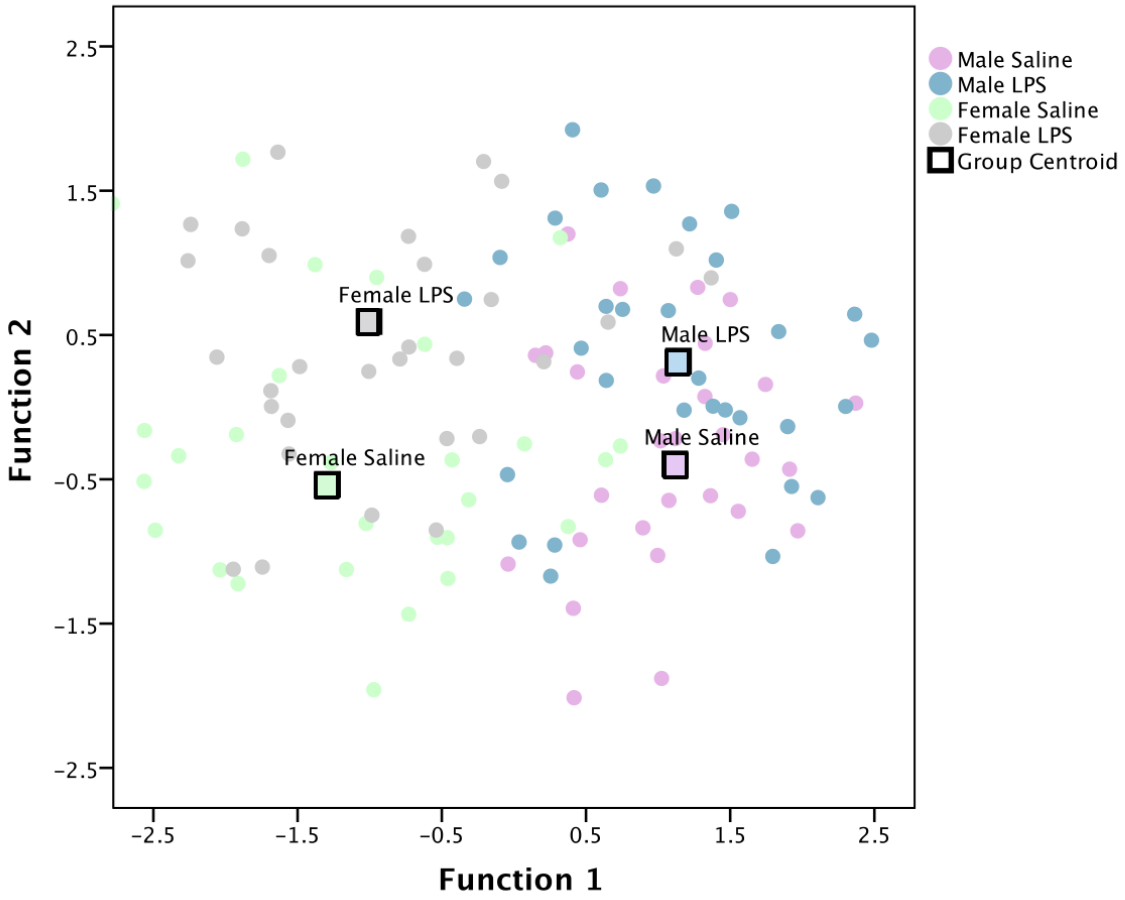

Figure 4.6 Canonical discriminant function plot for experiment 2 (sex × gavage treatment).

Experiment 2 discriminant function plot (sex by gavage treatment). Correlations between outcome variables and discriminant functions are listed in Table 4.2. Function 1 separates groups based on sex, largely driven by differences in time spent jumping, jump time, and ambulatory time, whereas function 2 separates groups by gavage treatment, mostly driven by number of rears, zone entries, and stereotypic counts. LPS affects males and females in a similar fashion across discriminant function 2. Group

centroids indicate the mean discriminant function value of each of the designated groups.

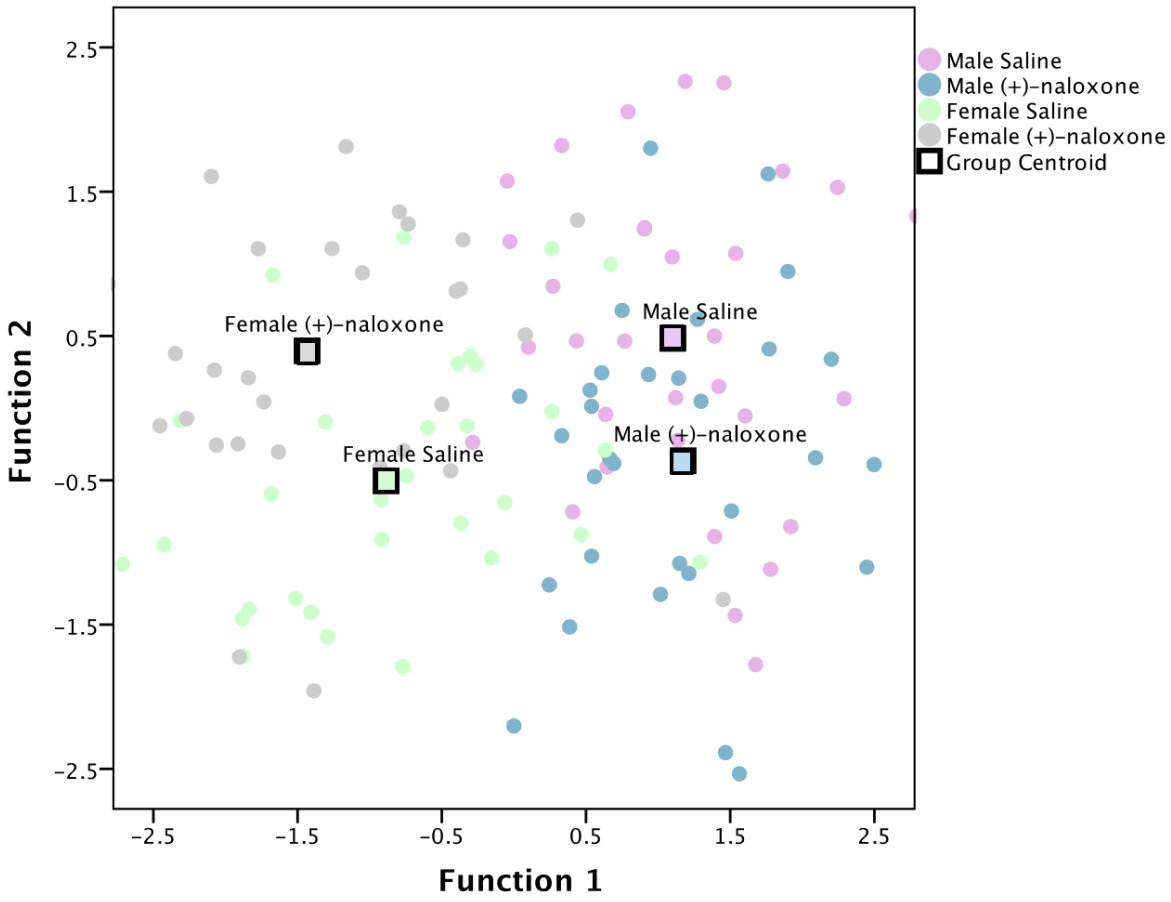

Figure 4.7 Canonical discriminant function plot for experiment 2 (sex × (+)-naloxone treatment).

Experiment 2 discriminant function plot (sex by (+)-naloxone treatment). Correlations between outcome variables and discriminant functions are listed in Table 4.3. Function 1 separates groups based on sex, largely driven by differences in time spent jumping, jump time, and ambulatory time, whereas function 2 demonstrates an interaction

between sex and (+)-naloxone treatment, mostly based on the sexually differential effect of (+)-naloxone on stereotypic counts, ambulatory episodes, and time spent in stereotypic circling. Group centroids indicate the mean discriminant function value of each of the designated groups.

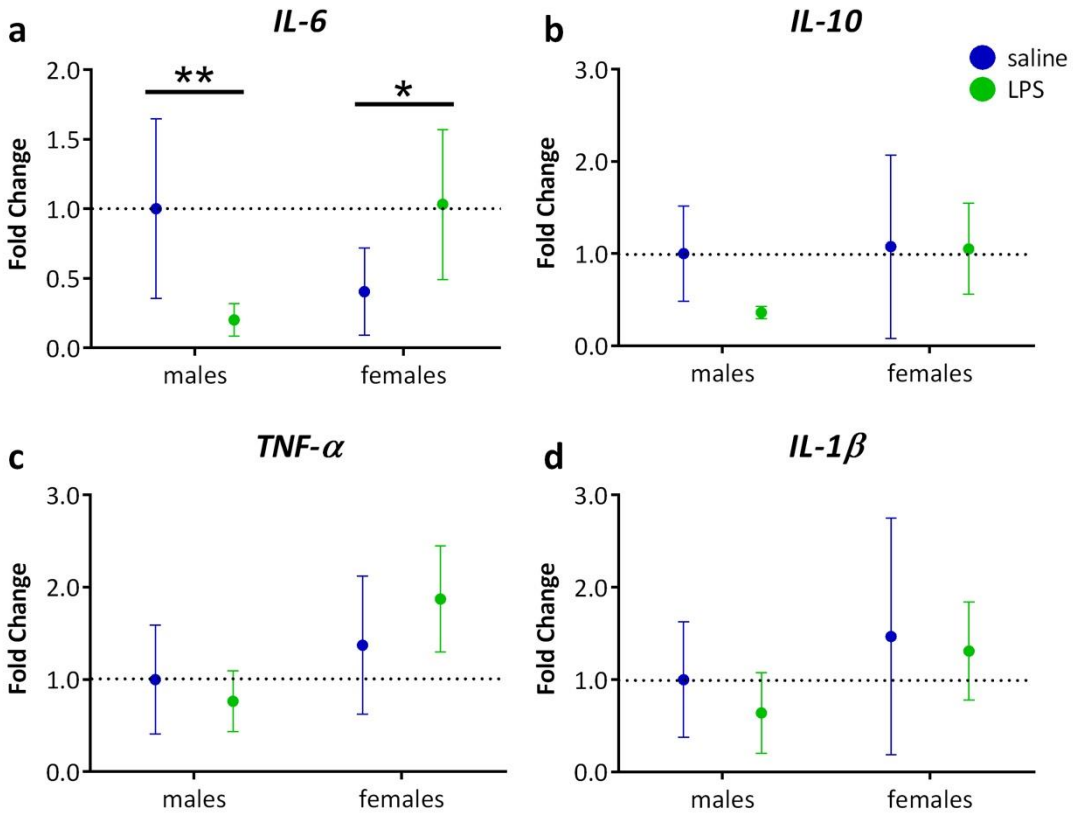

Figure 4.8 Effects of oral gavage of LPS on gut cytokine expression in female and male mice.

a *Expression levels of IL-6 showed a sex-dependent effect, with LPS causing a reduction of IL-6 in males and an increase in females.* ***b–d*** *However, the expression of IL-10, TNF-α, and IL-1β did not depend on sex or experimental treatment. Data are*

*expressed relative to levels of saline-treated males. Error bars are 95% confidence intervals. *p < 0.05; **p < 0.01*

Meta-analyses across Experiments 1 and 2

A random effects meta-analysis was performed across experimental cohorts in order to obtain more general estimates of the effects of oral gavage of LPS on behavior. As both experiments used WT males, all WT males from Experiment 1 and WT males not treated with TLR4 antagonists in Experiment 2 were used for the meta analyses (total n=15/group). Across Experiments 1 and 2, there were significant effects of LPS on time spent in the center zone (p<0.01), time spent in stereotypic circling (p<0.01), and stereotypic counts (p<0.01) (**Figure 4.9**). There were non-significant effects of LPS on parameters of locomotion, including ambulatory counts (p>0.1), ambulatory episodes (p>0.10), ambulatory time (p>0.1), ambulatory distance (p>0.10), resting time (p>0.10), and average velocity (p>0.10). All other behavioral parameters were also non-significant (**Supplemental Fig. 2-12, Supplemental Table 6**).

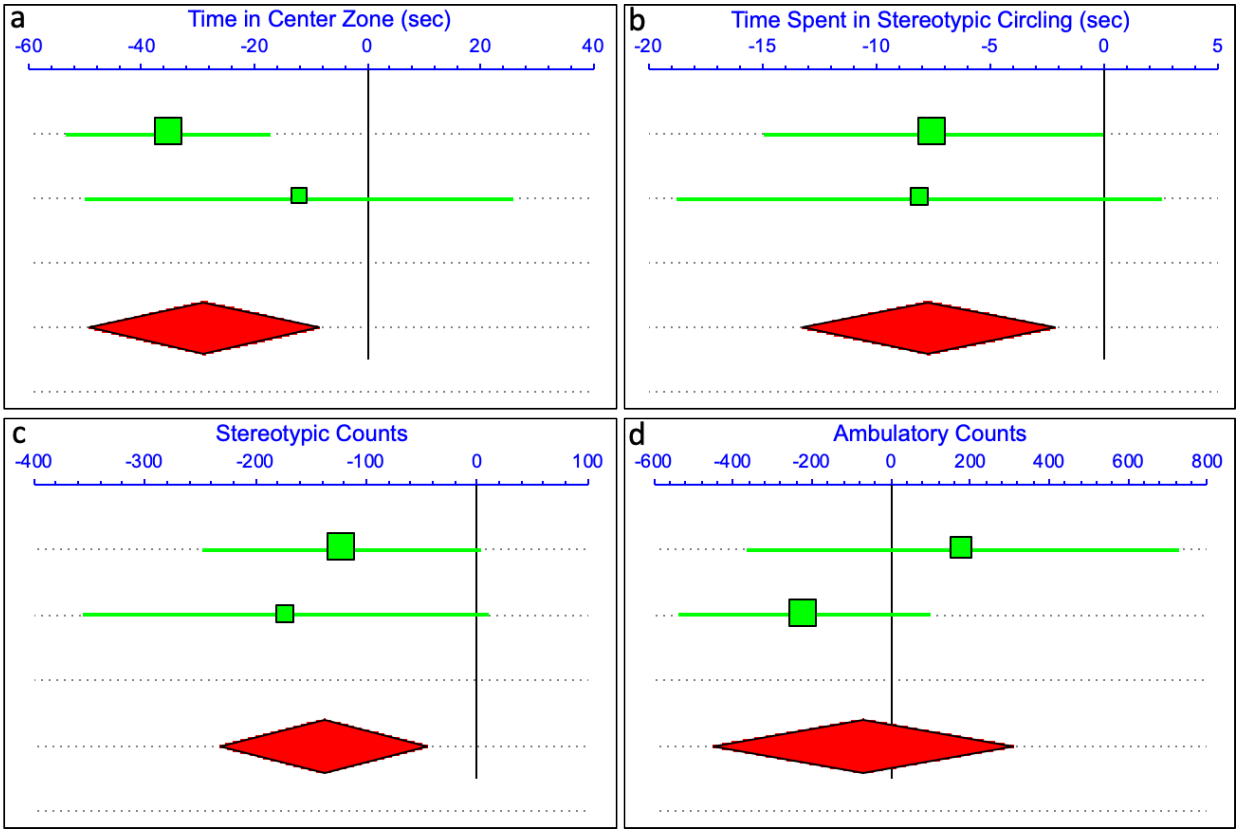

Figure 4.9 Meta-analyses of behavior in WT males of experiments 1 and 2.

*Forest plots of meta-analyses of **a** time spent in center zone, **b** time spent in stereotypic circling, **c** stereotypic counts, and **d** ambulatory counts measured in experiment 1 (top green bar) and experiment 2 (bottom green bar). The result of the meta-analysis is indicated by the red diamond. The width of the green bars and the red diamond indicate the range of the 95% confidence intervals for each, with the center representing the mean. WT males not treated with TLR4 antagonists were used for these analyses (total n = 15/group). See Supplemental Table 7 for statistics.*

4.5 Discussion

Here, we observe that gut-derived LPS elicits various aspects of the canonical sickness behavior response, with the exception of lethargy. In our first experiment, LPS increased anxiety-like behavior in male WT mice whereas no such effect was found in male *Tlr4-/-* mice. In the second experiment, LPS similarly increased anxiety-like behaviors in WT males and females. Neither TLR4 antagonist ((+)-naloxone nor LPS-RS) blocked the effects of gavage treatment. However, (+)-naloxone, a TLR4/TRIF specific antagonist, which does not interact with opioid receptors (Iijima et al., 1978; Marcoli et al., 1989), affected behavior differently in males and females. Furthermore, LPS-RS did not significantly alter behavior, suggesting that the MyD88 pathway may not be involved in anxiety-like and repetitive behaviors generated by gut-derived endotoxin. With the presented data, we offer oral administration of LPS as a model of gut dysbiosis that may result from overgrowth of pathogenic gram-negative bacteria in gut microbiota.

While LPS increased anxiety behavior two hours after treatment, it did not increase lethargy (indexed as hypolocomotion), as seen 2-6 hours after systemic injections of LPS. Importantly, oral gavage of LPS induced similar increases in anxiety-like behavior to those observed after direct injection (Lacosta et al., 1999; Swiergiel and Dunn, 2007; Juszczak et al., 2008; Painsipp et al., 2008; Zager et al., 2009; Haba et al., 2012; Sulakhiya et al., 2015; Ghisoni et al., 2016; Sulakhiya et al., 2016; Mayerhofer et al., 2017; Zager et al., 2017). This suggests that oral gavage of LPS specifically induced anxiety-like behavior without inducing a generalized sickness response.

In Experiment 1, an oral gavage of LPS increased anxiety-like behaviors in male WT mice, as measured by decreased time spent in the center zone of the open field

test. In Experiment 2, the oral gavage of LPS did not strongly affect time spent in the center zone in subject mice. This may be a result of increased anxiety stemming from the additional manipulations (e.g., intraperitoneal injection) in this experiment as both gavage and injections can increase anxiety (Meijer et al., 2006; Hoggatt et al., 2010) or from behavioral variability across experimental cohorts. Nevertheless, multivariate analyses from Experiment 2 indicate that gut-derived LPS produced a syndrome of behavioral alterations that includes increases in anxiety-like behaviors (increased incidence of vertical stretch posture and decreased zone entries) and decreases in repetitive behaviors (decreased jump time and jump counts), albeit along a slightly different combination of measures from that found in Experiment 1. In support of the conclusion from the multivariate analyses that LPS affects anxiety-like behaviors in both experiments, meta-analysis of LPS effects in WT males indicates that the observed reduction in time spent in the center zone, a highly-used index of anxiety-like behavior(Calabrese, 2008; Campos et al., 2013), is similar to the reported range of reduced time spent in the center zone for male mice injected intraperitoneally or intravenously with LPS (20 to 60 second difference per 5 minute segment) (Lacosta et al., 1999; Swiergiel and Dunn, 2007; Juszczak et al., 2008; Painsipp et al., 2008; Zager et al., 2009; Haba et al., 2012; Sulakhiya et al., 2015; Ghisoni et al., 2016; Sulakhiya et al., 2016; Mayerhofer et al., 2017; Zager et al., 2017). Overall, these data demonstrate the utility of multivariate analyses to highlight similar behavioral effects across differing contexts.

Our data indicate that the behavioral effects of gut-derived LPS are mediated through TLR4. Oral administration of LPS significantly increased anxiety-like behavior in

WT mice, but not in *Tlr4-/-* mice. If anything, there was a trend toward LPS increasing time spent in the center zone in *Tlr4-/-* mice, suggesting that LPS may interact with other innate immune receptors to decrease anxiety. TLR4 antagonists, however, did not directly block the effects of LPS gavage on behavior. It is unlikely that this is due to ineffective dosage, as we chose dosages of antagonists based on the literature (Sorge et al., 2011; Wu et al., 2012; Corrigan et al., 2015; Li et al., 2015a), and (+)-naloxone affected behavior regardless of LPS treatment in this study. Measurement of cytokines suggests that LPS acted primarily at the level of the gut, as we did not find elevation of inflammatory markers in serum but did find a significant elevation of IL-6 expression in the gut. If so, it may be that the TLR4 antagonists did not intervene effectively at the site of action of the LPS. Orally-administered LPS likely interacts with TLR4 present on the apical surface of intestinal epithelial cells. It is plausible that our antagonists, when injected intraperitoneally, do not have sufficient access to these receptors.

Our data suggest that there may be sex differences in constitutive TLR4 activity and its downstream effects on locomotor and repetitive behaviors. The antagonist (+)-naloxone, which blocks the TLR4/TRIF signaling pathway, increased stereotypic circling time and ambulatory episodes in females while decreasing these behaviors in males, regardless of gavage treatment. This suggests that the TLR4/TRIF pathway differently modulates these behaviors in males and females. This is in line with literature that shows (+)-naloxone more effectively blocks TLR4-modulated nociception in female than in male rats (Doyle et al., 2017). In our study, oral LPS treatment increased intestinal IL-6 expression in females and suppressed it in males. As IL-6 expression depends on the

TLR4/TRIF pathway (Shen et al., 2008), it is possible that sex differences in this pathway contributed to sex differences in LPS effects on IL-6 observed in this study.

Our data also demonstrate that TLR4 activation may suppress repetitive behaviors. Genetic deletion of TLR4 in males and blockade of TLR4 signaling (with (+)-naloxone) in females both increase stereotypic circling. These effects may possibly be driven by the suppression of allergic-type (Th2-driven) immune profiles by TLR4, as *Tlr4-/-* mice are reported to show enhanced allergic responses (Bashir et al., 2004; Berin et al., 2006). In line with this prediction, a number of studies demonstrate that allergic-type immune profiles increase repetitive behaviors (Tuomisto, 1986; Mills et al., 2000; Mazmanian et al., 2005; Thomas et al., 2009; Nishino et al., 2013; Desbonnet et al., 2014; Balazsfi et al., 2015; Fodor et al., 2016). It is notable that the TLR4 antagonist (+)-naloxone decreased stereotypic circling in males, while enhancing it in females. There are documented sex differences in cytokine responses to TLR4 activation (Santos-Galindo et al., 2011; Doyle et al., 2017). Our data further suggest potential sex differences in the TLR4/TRIF pathway in males and females, and these differences may contribute to the sex difference we observed in stereotypic circling among saline-treated mice. Alignment of the effects of TLR4 genetic mutation in males and effects of (+)-naloxone in females, and contrary effects of (+)-naloxone in males, suggest that TLR4 may play the same role in males and females, but the underlying signaling pathways may differ between the sexes.

In summary, in spite of the ubiquitous nature of LPS in the gut lumen, this is the first study to demonstrate that gut-derived LPS can initiate behavioral aspects of the sickness response. Our results suggest that an increased intestinal load of LPS similarly

increases anxiety-like behavior and suppresses repetitive behavior in males and females. However, to the extent this is mediated through TLR4 activation, this may occur via differing mechanisms. Furthermore, different actions of the TLR4/TRIF pathway may drive baseline differences in repetitive behaviors in males and females.

4.6 Supplemental Material

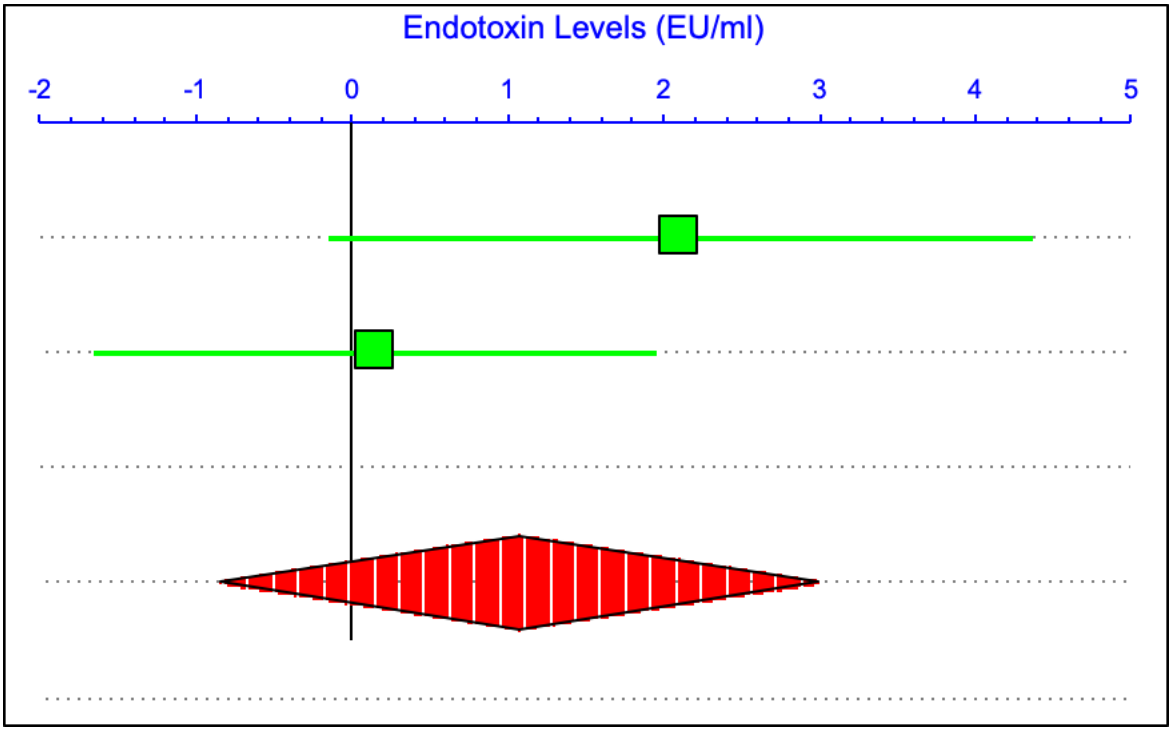

Figure 4.10 Supplemental Figure 1. Meta-analysis of serum endotoxin levels in WT males of Experiments 1 and 2.

Forest plot of difference in serum endotoxin levels between male WT subjects gavaged with saline or LPS, measured from Experiment 1 (top green bar) and Experiment 2 (bottom green bar). The result of the meta-analysis is indicated by the red diamond. The width of the green bars and the red diamond indicate the range of the 95% confidence

intervals for each, with the center representing the mean. WT males not treated with TLR4 antagonists were used for these analyses (total n = 15/group).

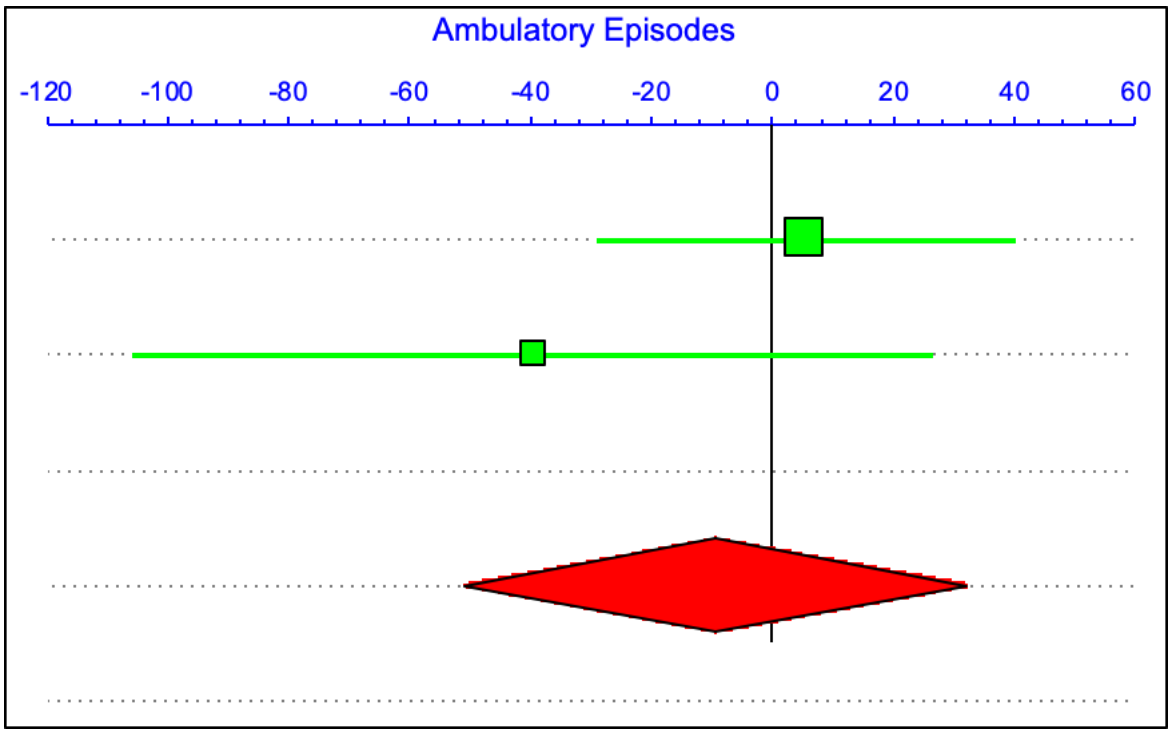

Figure 4.11 Supplemental Figure 2. Meta-analysis of ambulatory episodes in WT males of Experiments 1 and 2.

Forest plot of difference in ambulatory episodes between male WT subjects gavaged with saline or LPS, measured from Experiment 1 (top green bar) and Experiment 2 (bottom green bar). The result of the meta-analysis is indicated by the red diamond. The width of the green bars and the red diamond indicate the range of the 95% confidence intervals for each, with the center representing the mean. WT males not treated with TLR4 antagonists were used for these analyses (total n *= 15/group). See Supplemental Table 7 for statistics.*

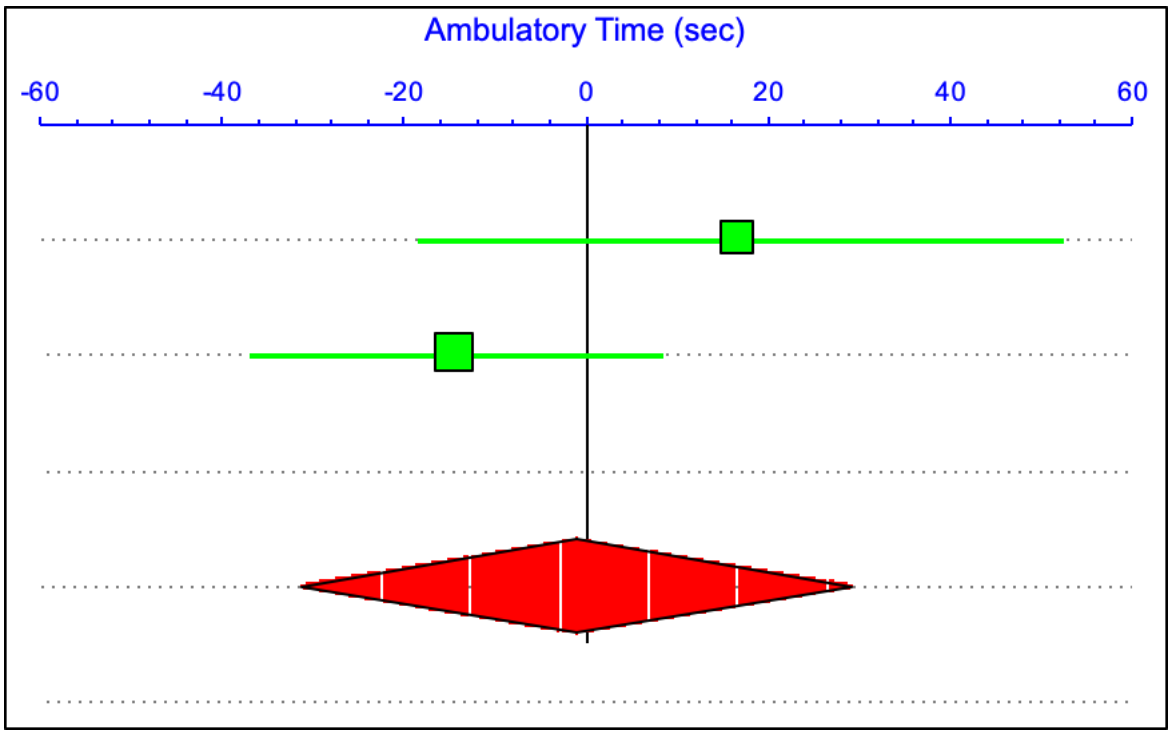

Figure 4.12 Supplemental Figure 3. Meta-analysis of ambulatory time in WT males of Experiments 1 and 2.

Forest plot of difference in ambulatory time between male WT subjects gavaged with saline or LPS, measured from Experiment 1 (top green bar) and Experiment 2 (bottom green bar). The result of the meta-analysis is indicated by the red diamond. The width of the green bars and the red diamond indicate the range of the 95% confidence intervals for each, with the center representing the mean. WT males not treated with TLR4 antagonists were used for these analyses (total n *= 15/group). See Supplemental Table 7 for statistics.*

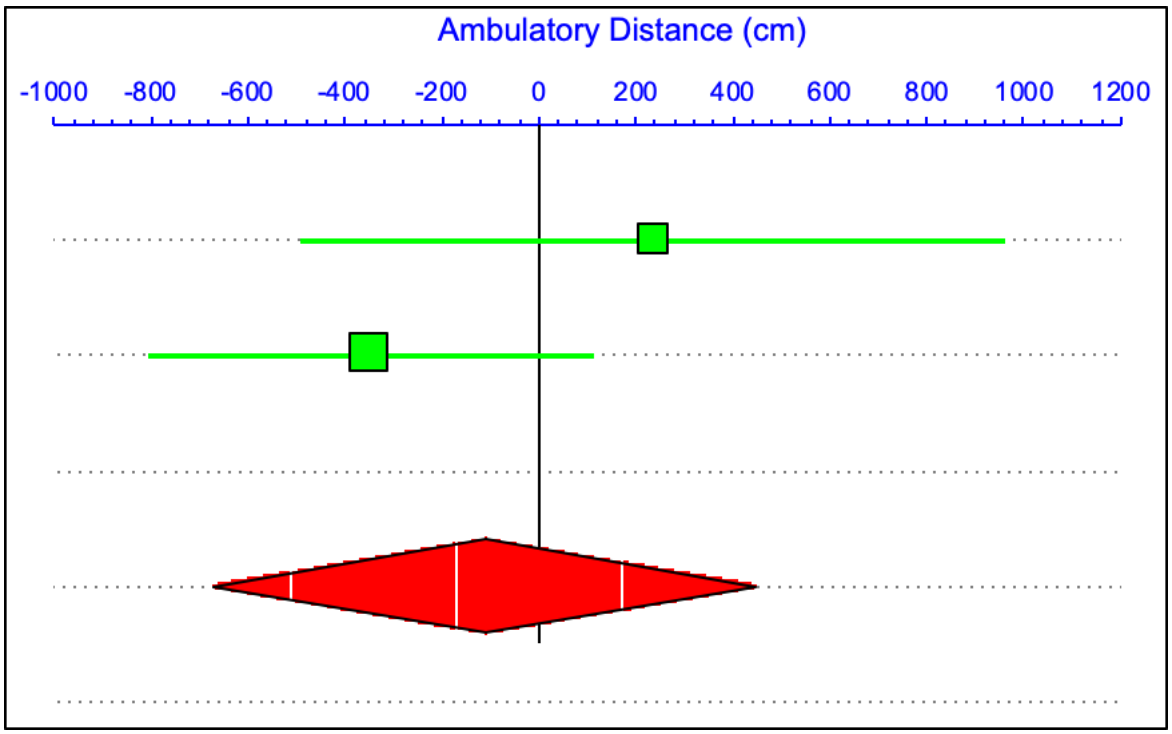

Figure 4.13 Supplemental Figure 4. Meta-analysis of ambulatory distance in WT males of Experiments 1 and 2.

Forest plot of difference in ambulatory distance between male WT subjects gavaged with saline or LPS, measured from Experiment 1 (top green bar) and Experiment 2 (bottom green bar). The result of the meta-analysis is indicated by the red diamond. The width of the green bars and the red diamond indicate the range of the 95% confidence intervals for each, with the center representing the mean. WT males not treated with TLR4 antagonists were used for these analyses (total n *= 15/group). See Supplemental Table 7 for statistics.*

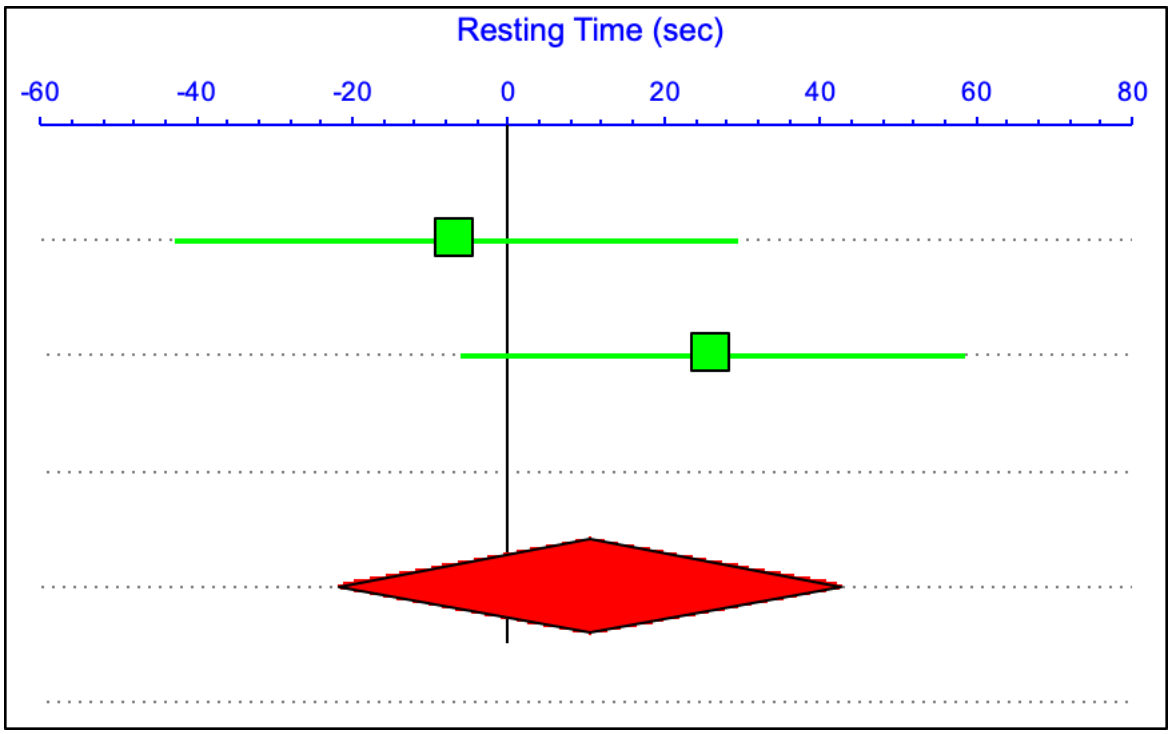

Figure 4.14 Supplemental Figure 5. Meta-analysis of resting time in WT males of Experiments 1 and 2.

Forest plot of difference in resting time between male WT subjects gavaged with saline or LPS, measured from Experiment 1 (top green bar) and Experiment 2 (bottom green bar). The result of the meta-analysis is indicated by the red diamond. The width of the green bars and the red diamond indicate the range of the 95% confidence intervals for each, with the center representing the mean. WT males not treated with TLR4 antagonists were used for these analyses (total n *= 15/group). See Supplemental Table 7 for statistics.*

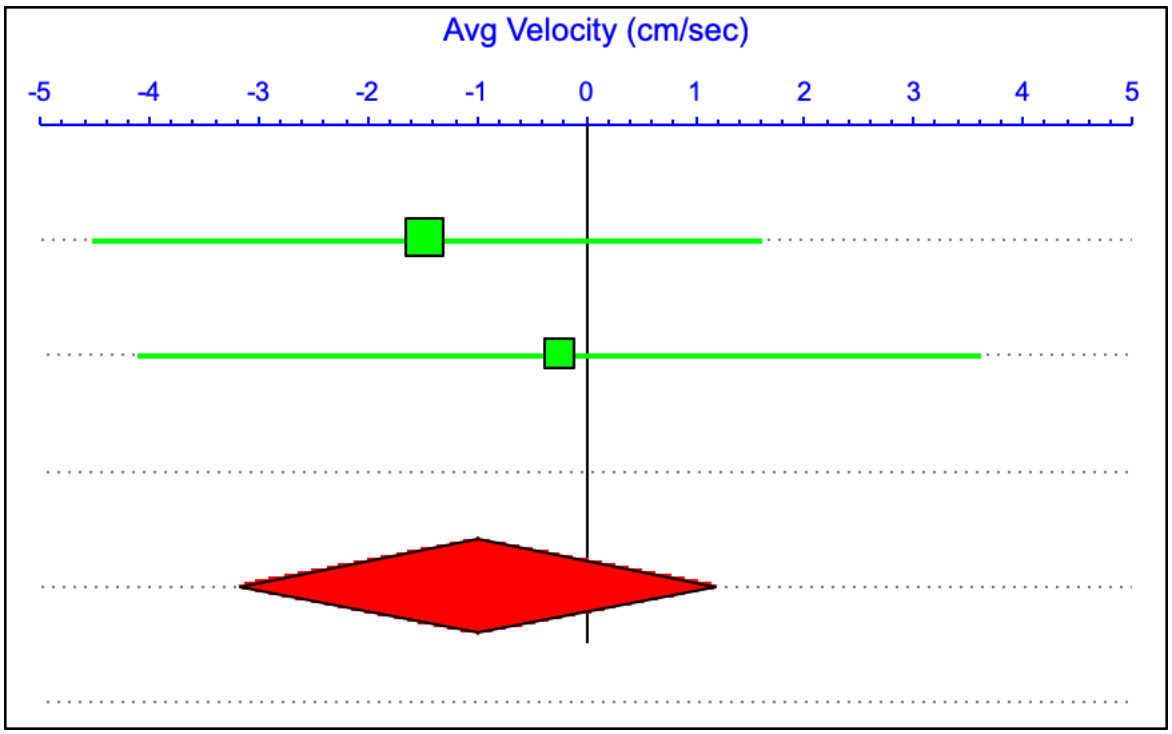

Figure 4.15 Supplemental Figure 6. Meta-analysis of average velocity in WT males of Experiments 1 and 2.

Forest plot of difference in average velocity between male WT subjects gavaged with saline or LPS, measured from Experiment 1 (top green bar) and Experiment 2 (bottom green bar). The result of the meta-analysis is indicated by the red diamond. The width of the green bars and the red diamond indicate the range of the 95% confidence intervals for each, with the center representing the mean. WT males not treated with TLR4 antagonists were used for these analyses (total n *= 15/group). See Supplemental Table 7 for statistics.*

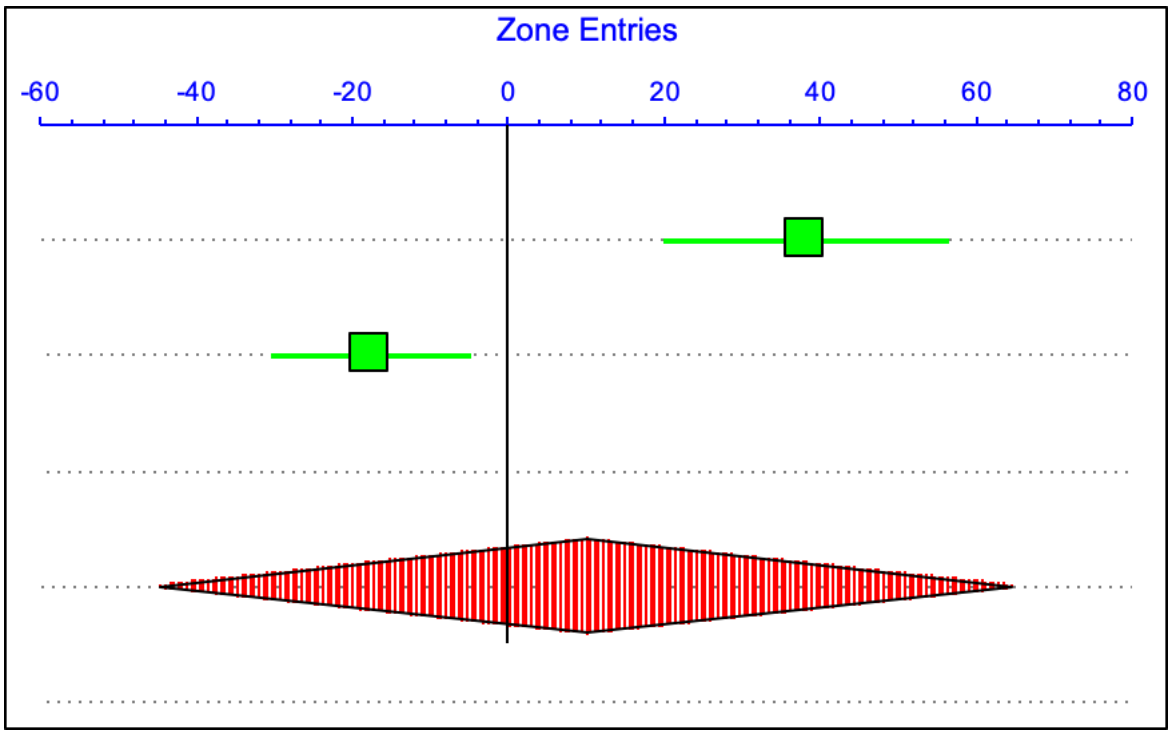

Figure 4.16 Supplemental Figure 7. Meta-analysis of zone entries in WT males of Experiments 1 and 2.

Forest plot of difference in zone entries between male WT subjects gavaged with saline or LPS, measured from Experiment 1 (top green bar) and Experiment 2 (bottom green bar). The result of the meta-analysis is indicated by the red diamond. The width of the green bars and the red diamond indicate the range of the 95% confidence intervals for each, with the center representing the mean. WT males not treated with TLR4 antagonists were used for these analyses (total n *= 15/group). See Supplemental Table 7 for statistics.*

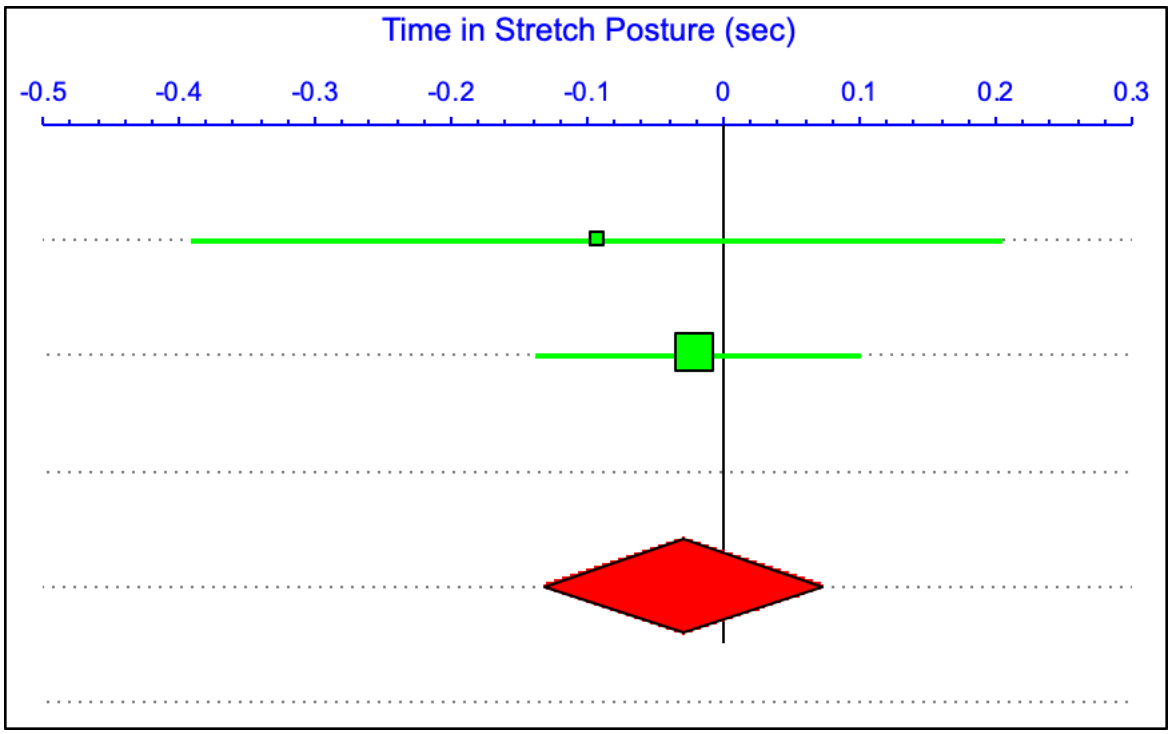

Figure 4.17 Supplemental Figure 8. Meta-analysis of stretch posture in WT males of Experiments 1 and 2.

Forest plot of difference in time in stretch posture between male WT subjects gavaged with saline or LPS, measured from Experiment 1 (top green bar) and Experiment 2 (bottom green bar). The result of the meta-analysis is indicated by the red diamond. The width of the green bars and the red diamond indicate the range of the 95% confidence intervals for each, with the center representing the mean. WT males not treated with TLR4 antagonists were used for these analyses (total n *= 15/group). See Supplemental Table 7 for statistics.*

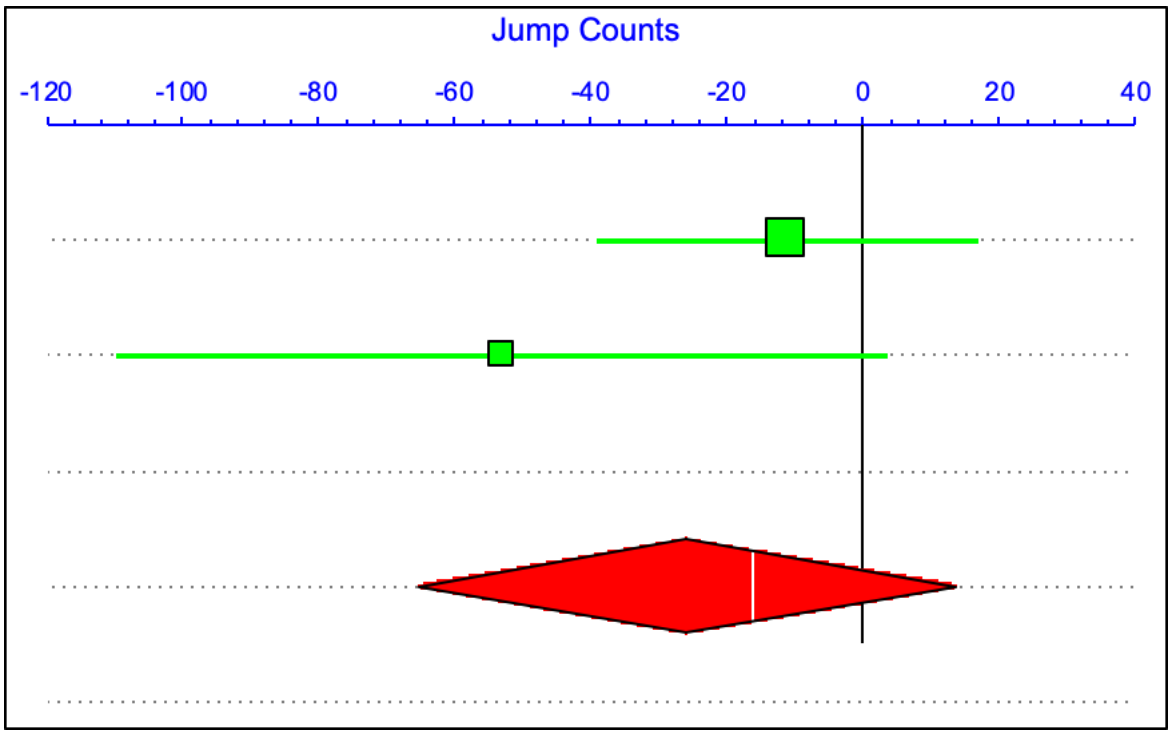

Figure 4.18 Supplemental Figure 9. Meta-analysis of jump counts in WT males of Experiments 1 and 2.

Forest plot of difference in jump counts between male WT subjects gavaged with saline or LPS, measured from Experiment 1 (top green bar) and Experiment 2 (bottom green bar). The result of the meta-analysis is indicated by the red diamond. The width of the green bars and the red diamond indicate the range of the 95% confidence intervals for each, with the center representing the mean. WT males not treated with TLR4 antagonists were used for these analyses (total n *= 15/group). See Supplemental Table 7 for statistics.*

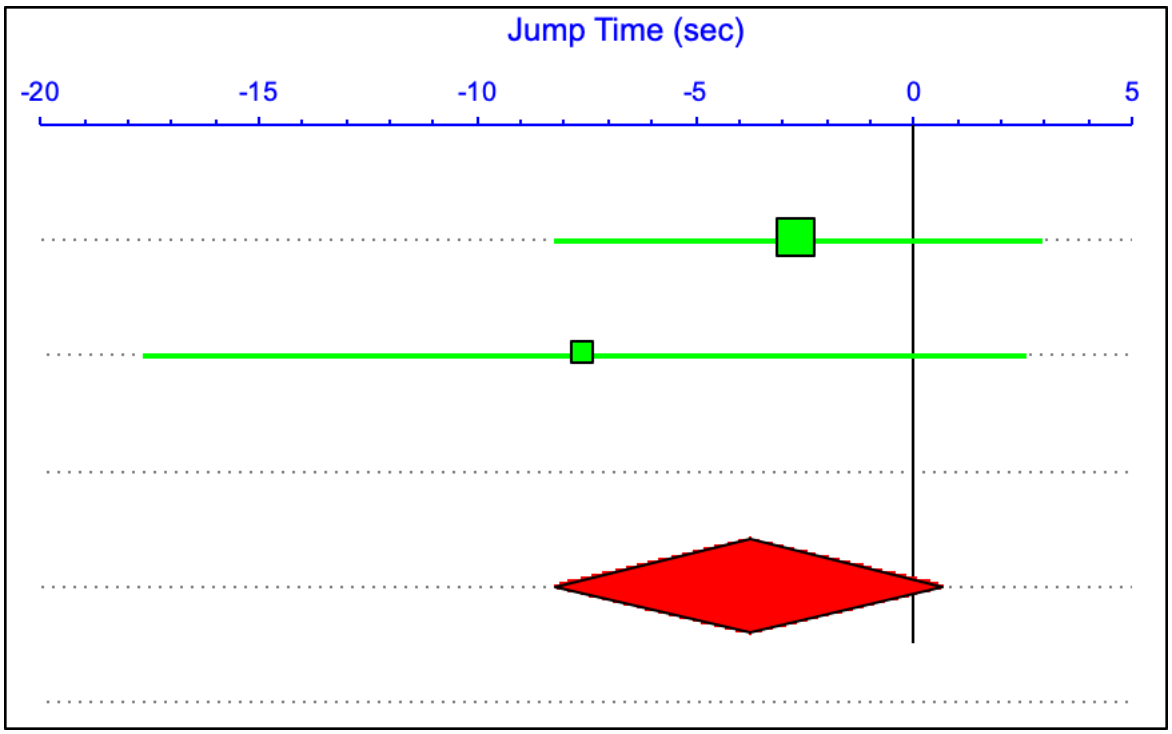

Figure 4.19 Supplemental Figure 10. Meta-analysis of jump time in WT males of Experiments 1 and 2.

Forest plot of difference in jump time between male WT subjects gavaged with saline or LPS, measured from Experiment 1 (top green bar) and Experiment 2 (bottom green bar). The result of the meta-analysis is indicated by the red diamond. The width of the green bars and the red diamond indicate the range of the 95% confidence intervals for each, with the center representing the mean. WT males not treated with TLR4 antagonists were used for these analyses (total n = 15/group). See Supplemental Table 7 for statistics.

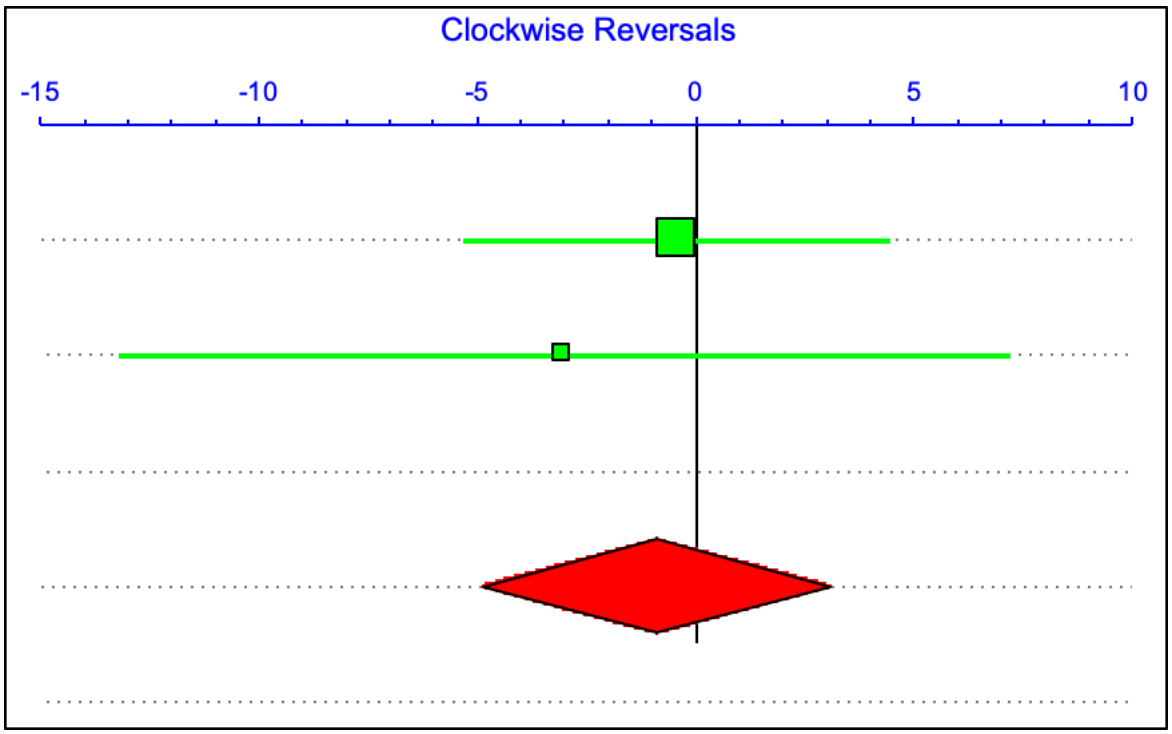

Figure 4.20 Supplemental Figure 11. Meta-analysis of clockwise reversals in WT males of Experiments 1 and 2.

Forest plot of difference in clockwise reversals between male WT subjects gavaged with saline or LPS, measured from Experiment 1 (top green bar) and Experiment 2 (bottom green bar). The result of the meta-analysis is indicated by the red diamond. The width of the green bars and the red diamond indicate the range of the 95% confidence intervals for each, with the center representing the mean. WT males not treated with TLR4 antagonists were used for these analyses (total n = 15/group). See Supplemental Table 7 for statistics.

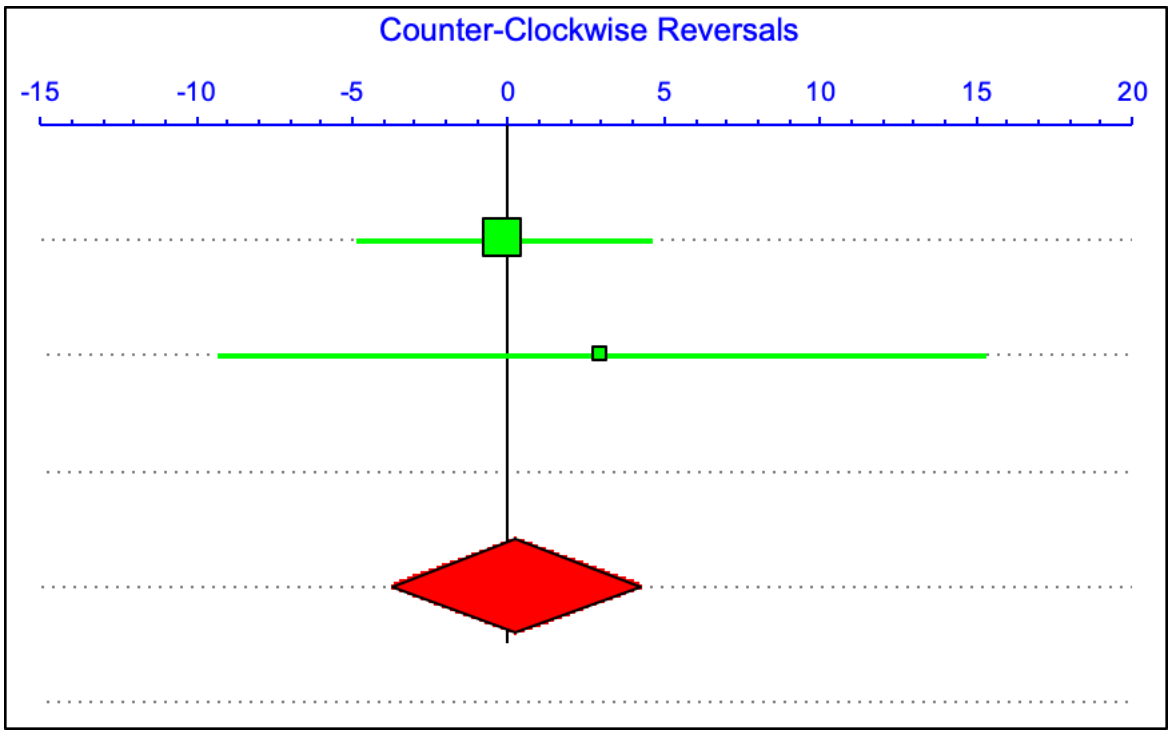

Figure 4.21 Supplemental Figure 12. Meta-analysis of counter-clockwise reversals in WT males of Experiments 1 and 2.

Forest plot of difference in counter-clockwise reversals between male WT subjects gavaged with saline or LPS, measured from Experiment 1 (top green bar) and Experiment 2 (bottom green bar). The result of the meta-analysis is indicated by the red diamond. The width of the green bars and the red diamond indicate the range of the 95% confidence intervals for each, with the center representing the mean. WT males not treated with TLR4 antagonists were used for these analyses (total n = 15/group). See Supplemental Table 7 for statistics.

Table 4.4 Supplemental Table 1. Independent ANOVAs from Experiment 1 suggest outcome variables that contribute to group differences highlighted by Pillai's trace.

Source	Dependent Variable	F	Sig.	Partial η^2
Gavage Treatment	Time in Center Zone	0.25	0.623	0.014
	Number of Center Zone Entries	0.040	0.843	0.002
	Time Spent in Stereotypic Circling	0.489	0.493	0.026
	Number of Clockwise Reversals	0.459	0.507	0.025
	Time Spent in Vertical Stretch Posture	0.32	0.579	0.017
	Jump Counts	0.078	0.783	0.004
	Number of Counterclockwise Reversals	0.032	0.86	0.002
	Time Spent Jumping	0.08	0.781	0.004
	Incidence of Vertical Stretch Posture	.	.	.
	Ambulatory Episodes	0.327	0.574	0.018
	Ambulatory Counts	0.393	0.539	0.021
	Ambulatory Distance	0.229	0.638	0.013
Genotype	**Time in Center Zone**	**10.742**	**0.004**	**0.374**
	Number of Center Zone Entries	1.857	0.190	0.094
	Time Spent in Stereotypic Circling	**40.597**	**0**	**0.693**
	Number of Clockwise Reversals	0.068	0.797	0.004

	Time Spent in Vertical Stretch Posture	1.564	0.227	0.08
	Jump Counts	1.813	0.195	0.091
	Number of Counterclockwise Reversals	1.197	0.288	0.062
	Time Spent Jumping	**24.089**	**0**	**0.572**
	Incidence of Vertical Stretch Posture	.	.	.
	Ambulatory Episodes	0.302	0.589	0.017
	Ambulatory Counts	0.591	0.452	0.032
	Ambulatory Distance	0.168	0.687	0.009
Gavage Treatment	**Time in Center Zone**	**14.051**	**0.001**	**0.438**
by Genotype	Numbe of Center Zone Entries	0.865	0.365	0.046
	Time Spent in Stereotypic Circling	2.187	0.156	0.108
	Number of Clockwise Reversals	0.86	0.366	0.046
	Time Spent in Vertical Stretch Posture	0.106	0.749	0.006
	Jump Counts	0.682	0.42	0.036
	Number of Counterclockwise Reversals	0.01	0.922	0.001
	Time Spent Jumping	1.231	0.282	0.064
	Incidence of Vertical Stretch Posture	.	.	.
	Ambulatory Episodes	0.018	0.894	0.001

Ambulatory Counts	0.121	0.732	0.007
Ambulatory Distance	0.216	0.647	0.012

Individual ANOVAs on outcome variables measured in Experiment 1. Significant results are boldfaced. F values are indicated in the "F" column, p values are indicated in the "Sig." column and effect sizes (partial eta squared) are indicated in the "Partial η^2" column. For each ANOVA, hypothesis degrees of freedom is 1 and error degrees of freedom is 18.

Table 4.5 Supplemental Table 2. Original classification and cross-validation of discriminant functions for Experiment 1.

			Predicted Group Membership				
		Complete Label Code	WT saline	WT LPS	Tlr4-/- saline	Tlr4-/- LPS	Total
Original	Count	WT saline	7	0	0	0	7
		WT LPS	0	7	0	0	7
		Tlr4-/- saline	0	0	4	0	4
		Tlr4-/- LPS	0	0	0	4	4
	%	WT saline	100.0	.0	.0	.0	100.0
		WT LPS	.0	100.0	.0	.0	100.0
		Tlr4-/- saline	.0	.0	100.0	.0	100.0
		Tlr4-/- LPS	.0	.0	.0	100.0	100.0
Cross-validated	Count	WT saline	4	0	0	3	7
		WT LPS	0	6	0	1	7

		Tlr4-/- saline	0	0	2	2	4
		Tlr4-/- LPS	1	0	1	2	4
	%	WT saline	57.1	.0	.0	42.9	100.0
		WT LPS	.0	85.7	.0	14.3	100.0
		Tlr4-/- saline	.0	.0	50.0	50.0	100.0
		Tlr4-/- LPS	25.0	.0	25.0	50.0	100.0

Validation of discriminant functions for Experiment 1 by original case classification and leave-one-out cross validation. 100% of the original grouped cases are correctly classified by the discriminant functions. In the leave-one-out cross-validation test, the discriminant functions are recalculated excluding one case, and all cases are recalculated. This algorithm is repeated for the exclusion of each case. In the leave-one-out test, 63.6% of cross-validated grouped cases were correctly classified.

Table 4.6 Supplemental Table 3. Independent ANOVAs from Experiment 2 suggest outcome variables that contribute to group differences highlighted by Pillai's trace.

Source	Dependent Variable	F	Sig.	Partial Eta Squared
Sex	**Jump Counts**	**62.354**	**0**	**0.36**
	Jump Time	**39.281**	**0**	**0.261**
	Ambulatory Episodes	**18.094**	**0**	**0.14**
	Ambulatory Counts	**21.666**	**0**	**0.163**

	Time Spent in Stereotypic Circling	1.47	0.228	0.013
	Incidence of Vertical Stretch Posture	0.003	0.96	0
	Time Spent in Vertical Stretch Posture	*2.795*	*0.097*	*0.025*
	Time in Center Zone	0.084	0.772	0.001
	Number of Center Zone Entries	0.71	0.401	0.006
	Number of Clockwise Reversals	0.255	0.614	0.002
	Number of Counter-Clockwise Reversals	1.746	0.189	0.015
	Ambulatory Distance	**19.035**	**0**	**0.146**
	Average Velocity	**4.76**	**0.031**	**0.041**
	Ambulatory Time	24.905	0	0.183
	Stereotypic Counts	**0.064**	**0.8**	**0.001**
	Resting Time	**19.284**	**0**	**0.148**
Gavage_Treatment	**Jump Counts**	**6.315**	**0.013**	**0.054**
	Jump Time	*3.611*	*0.06*	*0.032*
	Ambulatory Episodes	1.694	0.196	0.015
	Ambulatory Counts	*3.031*	*0.084*	*0.027*
	Time Spent in Stereotypic Circling	**3.911**	**0.05**	**0.034**

	Incidence of Vertical Stretch Posture	**4.645**	**0.033**	**0.04**
	Time Spent in Vertical Stretch Posture	1.982	0.162	0.018
	Time in Center Zone	0.601	0.44	0.005
	Number of Center Zone Entries	5.69	0.019	0.049
	Number of Clockwise Reversals	0.159	0.691	0.001
	Number of Counter-Clockwise Reversals	1.746	0.189	0.015
	Ambulatory Distance	*2.866*	*0.093*	*0.025*
	Average Velocity	1.242	0.267	0.011
	Ambulatory Time	1.684	0.197	0.015
	Stereotypic Counts	**5.114**	**0.026**	**0.044**
	Resting Time	*3.507*	*0.064*	*0.031*
Naloxone_Treatment	Jump Counts	1.224	0.271	0.011
	Jump Time	0.051	0.821	0
	Ambulatory Episodes	1.153	0.285	0.01
	Ambulatory Counts	1.457	0.23	0.013
	Time Spent in Stereotypic Circling	0.03	0.862	0
	Incidence of Vertical Stretch Posture	0.565	0.454	0.005

	Time Spent in Vertical Stretch Posture	1.982	0.162	0.018
	Time in Center Zone	0.109	0.742	0.001
	Number of Center Zone Entries	0.214	0.645	0.002
	Number of Clockwise Reversals	0.374	0.542	0.003
	Number of Counter-Clockwise Reversals	0.03	0.863	0
	Ambulatory Distance	1.572	0.213	0.014
	Average Velocity	1.04	0.31	0.009
	Ambulatory Time	1.228	0.27	0.011
	Stereotypic Counts	0.033	0.857	0
	Resting Time	0.673	0.414	0.006
LPSRS_Treatment	Jump Counts	0.818	0.368	0.007
	Jump Time	0.152	0.698	0.001
	Ambulatory Episodes	0.272	0.603	0.002
	Ambulatory Counts	0.223	0.638	0.002
	Time Spent in Stereotypic Circling	1.189	0.278	0.011
	Incidence of Vertical Stretch Posture	0.565	0.454	0.005
	Time Spent in Vertical Stretch Posture	0.031	0.861	0

	Time in Center Zone	1.932	0.167	0.017
	Number of Center Zone Entries	0.453	0.502	0.004
	Number of Clockwise Reversals	0.035	0.853	0
	Number of Counter-Clockwise Reversals	0.991	0.322	0.009
	Ambulatory Distance	0.313	0.577	0.003
	Average Velocity	2.918	0.09	0.026
	Ambulatory Time	0.52	0.472	0.005
	Stereotypic Counts	0.463	0.498	0.004
	Resting Time	0.656	0.42	0.006
Sex * Gavage_Treatment	Jump Counts	0.221	0.639	0.002
	Jump Time	0.051	0.822	0
	Ambulatory Episodes	0.831	0.364	0.007
	Ambulatory Counts	1.029	0.313	0.009
	Time Spent in Stereotypic Circling	0.015	0.902	0
	Incidence of Vertical Stretch Posture	2.113	0.149	0.019
	Time Spent in Vertical Stretch Posture	1.309	0.255	0.012
	Time in Center Zone	1.975	0.163	0.017

	Number of Center Zone Entries	0.414	0.521	0.004
	Number of Clockwise Reversals	0.159	0.691	0.001
	Number of Counter-Clockwise Reversals	0.288	0.593	0.003
	Ambulatory Distance	0.631	0.429	0.006
	Average Velocity	0.056	0.814	0.001
	Ambulatory Time	0.802	0.372	0.007
	Stereotypic Counts	0.015	0.904	0
	Resting Time	0.455	0.501	0.004
Sex * Naloxone_Treatment	**Jump Counts**	**7.133**	**0.009**	**0.06**
	Jump Time	2.137	0.147	0.019
	Ambulatory Episodes	**10.018**	**0.002**	**0.083**
	Ambulatory Counts	**7.888**	**0.006**	**0.066**
	Time Spent in Stereotypic Circling	**6.015**	**0.016**	**0.051**
	Incidence of Vertical Stretch Posture	2.113	0.149	0.019
	Time Spent in Vertical Stretch Posture	0.379	0.539	0.003
	Time in Center Zone	0.133	0.716	0.001
	Number of Center Zone Entries	0.069	0.793	0.001

	Number of Clockwise Reversals	0.968	0.327	0.009
	Number of Counter-Clockwise Reversals	0.374	0.542	0.003
	Ambulatory Distance	**7.525**	**0.007**	**0.063**
	Average Velocity	**5.315**	**0.023**	**0.046**
	Ambulatory Time	**7.326**	**0.008**	**0.062**
	Stereotypic Counts	**7.635**	**0.007**	**0.064**
	Resting Time	**9.058**	**0.003**	**0.075**
Sex * LPSRS_Treatment	Jump Counts	0.981	0.324	0.009
	Jump Time	0.122	0.728	0.001
	Ambulatory Episodes	0.723	0.397	0.006
	Ambulatory Counts	0.596	0.442	0.005
	Time Spent in Stereotypic Circling	0.188	0.665	0.002
	Incidence of Vertical Stretch Posture	2.113	0.149	0.019
	Time Spent in Vertical Stretch Posture	0.07	0.792	0.001
	Time in Center Zone	1.349	0.248	0.012
	Number of Center Zone Entries	0.099	0.754	0.001
	Number of Clockwise Reversals	0.595	0.442	0.005

	Number of Counter-Clockwise Reversals	0.15	0.699	0.001
	Ambulatory Distance	0.578	0.449	0.005
	Average Velocity	*2.819*	*0.096*	*0.025*
	Ambulatory Time	0.633	0.428	0.006
	Stereotypic Counts	0.27	0.604	0.002
	Resting Time	0.618	0.434	0.006
Gavage_Treatment * Naloxone_Treatment	Jump Counts	0.632	0.428	0.006
	Jump Time	0.611	0.436	0.005
	Ambulatory Episodes	0.023	0.879	0
	Ambulatory Counts	0.122	0.728	0.001
	Time Spent in Stereotypic Circling	0.141	0.708	0.001
	Incidence of Vertical Stretch Posture	0.425	0.516	0.004
	Time Spent in Vertical Stretch Posture	0.279	0.599	0.003
	Time in Center Zone	0.03	0.862	0
	Number of Center Zone Entries	0.075	0.784	0.001
	Number of Clockwise Reversals	0.68	0.411	0.006
	Number of Counter-Clockwise Reversals	0.15	0.699	0.001

	Ambulatory Distance	0.124	0.726	0.001
	Average Velocity	2.168	0.144	0.019
	Ambulatory Time	0.098	0.761	0.001
	Stereotypic Counts	0.119	0.731	0.001
	Resting Time	0.218	0.642	0.002
Gavage_Treatment * LPSRS_Treatment	Jump Counts	0.571	0.451	0.005
	Jump Time	0.376	0.541	0.003
	Ambulatory Episodes	0.44	0.509	0.004
	Ambulatory Counts	0.234	0.629	0.002
	Time Spent in Stereotypic Circling	0.222	0.638	0.002
	Incidence of Vertical Stretch Posture	0.425	0.516	0.004
	Time Spent in Vertical Stretch Posture	0	1	0
	Time in Center Zone	0.68	0.411	0.006
	Number of Center Zone Entries	1.392	0.241	0.012
	Number of Clockwise Reversals	0.68	0.411	0.006
	Number of Counter-Clockwise Reversals	0.918	0.34	0.008
	Ambulatory Distance	0.367	0.546	0.003

	Average Velocity	0.122	0.727	0.001
	Ambulatory Time	0.479	0.49	0.004
	Stereotypic Counts	0.157	0.693	0.001
	Resting Time	0.579	0.448	0.005
Naloxone_Treatment * LPSRS_Treatment	Jump Counts	0.02	0.887	0
	Jump Time	0.002	0.963	0
	Ambulatory Episodes	1.004	0.318	0.009
	Ambulatory Counts	0.431	0.513	0.004
	Time Spent in Stereotypic Circling	0.164	0.687	0.001
	Incidence of Vertical Stretch Posture	0.425	0.516	0.004
	Time Spent in Vertical Stretch Posture	0.031	0.861	0
	Time in Center Zone	0.174	0.677	0.002
	Number of Center Zone Entries	1.062	0.305	0.009
	Number of Clockwise Reversals	0.018	0.894	0
	Number of Counter-Clockwise Reversals	0.26	0.611	0.002
	Ambulatory Distance	0.305	0.582	0.003
	Average Velocity	0.611	0.436	0.005

	Ambulatory Time	0.642	0.425	0.006
	Stereotypic Counts	0.224	0.637	0.002
	Resting Time	0.538	0.465	0.005
Sex * Gavage_Treatment * Naloxone_Treatment	Jump Counts	1.846	0.177	0.016
	Jump Time	0.328	0.568	0.003
	Ambulatory Episodes	0.733	0.394	0.007
	Ambulatory Counts	1.066	0.304	0.01
	Time Spent in Stereotypic Circling	0.011	0.916	0
	Incidence of Vertical Stretch Posture	0.003	0.96	0
	Time Spent in Vertical Stretch Posture	0.379	0.539	0.003
	Time in Center Zone	0.369	0.545	0.003
	Number of Center Zone Entries	0.013	0.908	0
	Number of Clockwise Reversals	0.018	0.894	0
	Number of Counter-Clockwise Reversals	0.387	0.535	0.003
	Ambulatory Distance	0.675	0.413	0.006
	Average Velocity	0.199	0.656	0.002
	Ambulatory Time	0.611	0.436	0.005

	Stereotypic Counts	0.143	0.706	0.001
	Resting Time	0.421	0.518	0.004
Sex * Gavage_Treatment * LPSRS_Treatment	Jump Counts	0.634	0.427	0.006
	Jump Time	0.771	0.382	0.007
	Ambulatory Episodes	0.531	0.468	0.005
	Ambulatory Counts	0.351	0.555	0.003
	Time Spent in Stereotypic Circling	0.082	0.775	0.001
	Incidence of Vertical Stretch Posture	0.003	0.96	0
	Time Spent in Vertical Stretch Posture	0.194	0.661	0.002
	Time in Center Zone	0.757	0.386	0.007
	Number of Center Zone Entries	0.001	0.981	0
	Number of Clockwise Reversals	0.018	0.894	0
	Number of Counter-Clockwise Reversals	0.329	0.567	0.003
	Ambulatory Distance	0.448	0.505	0.004
	Average Velocity	0.172	0.679	0.002
	Ambulatory Time	0.409	0.524	0.004
	Stereotypic Counts	0.007	0.932	0

	Resting Time	0.287	0.593	0.003
Sex * Naloxone_Treatment * LPSRS_Treatment	Jump Counts	0.132	0.717	0.001
	Jump Time	0.034	0.854	0
	Ambulatory Episodes	0.351	0.555	0.003
	Ambulatory Counts	0.16	0.689	0.001
	Time Spent in Stereotypic Circling	0.611	0.436	0.005
	Incidence of Vertical Stretch Posture	1.831	0.179	0.016
	Time Spent in Vertical Stretch Posture	0.379	0.539	0.003
	Time in Center Zone	0.009	0.925	0
	Number of Center Zone Entries	3.993	0.048	0.035
	Number of Clockwise Reversals	0.595	0.442	0.005
	Number of Counter-Clockwise Reversals	1.204	0.275	0.011
	Ambulatory Distance	0.133	0.716	0.001
	Average Velocity	0.046	0.83	0
	Ambulatory Time	0.175	0.677	0.002
	Stereotypic Counts	0.432	0.512	0.004
	Resting Time	0.336	0.564	0.003

Gavage_Treatment * Naloxone_Treatment * LPSRS_Treatment	Jump Counts	1.445	0.232	0.013
	Jump Time	1	0.32	0.009
	Ambulatory Episodes	1.24	0.268	0.011
	Ambulatory Counts	0.376	0.541	0.003
	Time Spent in Stereotypic Circling	2.32	0.131	0.02
	Incidence of Vertical Stretch Posture	0.565	0.454	0.005
	Time Spent in Vertical Stretch Posture	1.115	0.293	0.01
	Time in Center Zone	0.117	0.733	0.001
	Number of Center Zone Entries	0.133	0.716	0.001
	Number of Clockwise Reversals	0.204	0.652	0.002
	Number of Counter-Clockwise Reversals	0.104	0.747	0.001
	Ambulatory Distance	0.697	0.406	0.006
	Average Velocity	2.653	0.106	0.023
	Ambulatory Time	0.503	0.48	0.005
	Stereotypic Counts	2.499	0.117	0.022
	Resting Time	1.323	0.253	0.012

Sex *	Jump Counts	0	0.994	0
Gavage_Treatment *	Jump Time	0.159	0.691	0.001
Naloxone_Treatment *	Ambulatory Episodes	0.859	0.356	0.008
LPSRS_Treatment	Ambulatory Counts	0.51	0.477	0.005
	Time Spent in Stereotypic Circling	1.305	0.256	0.012
	Incidence of Vertical Stretch Posture	0.003	0.96	0
	Time Spent in Vertical Stretch Posture	*3.415*	*0.067*	*0.03*
	Time in Center Zone	0.009	0.926	0
	Number of Center Zone Entries	0.759	0.386	0.007
	Number of Clockwise Reversals	1.189	0.278	0.011
	Number of Counter-Clockwise Reversals	0.13	0.719	0.001
	Ambulatory Distance	0.654	0.42	0.006
	Average Velocity	0.01	0.921	0
	Ambulatory Time	0.889	0.348	0.008
	Stereotypic Counts	1.368	0.245	0.012
	Resting Time	1.248	0.266	0.011

Individual ANOVAs on outcome variables measured in Experiment 2. Significant results are boldfaced. Trends are italicized. F values are indicated in the "F" column, p values are indicated in the "Sig." column and effect sizes (partial eta squared) are indicated in the "Partial η^2" column. For each ANOVA, hypothesis degrees of freedom is 1 and error degrees of freedom is 111.

Table 4.7 Supplemental Table 4. Original classification and cross-validation of discriminant functions for Experiment 2 for cases grouped by gavage treatment and sex.

			Predicted Group Membership				
		SexByGavage	Male Saline	Male LPS	Female Saline	Female LPS	Total
Original	Count	Male Saline	22	9	1	0	32
		Male LPS	9	20	1	2	32
		Female Saline	3	2	21	5	31
		Female LPS	0	4	9	19	32
	%	Male Saline	68.8	28.1	3.1	.0	100.0
		Male LPS	28.1	62.5	3.1	6.3	100.0
		Female Saline	9.7	6.5	67.7	16.1	100.0
		Female LPS	.0	12.5	28.1	59.4	100.0
Cross-validated	Count	Male Saline	15	13	3	1	32
		Male LPS	13	14	2	3	32
		Female Saline	5	3	14	9	31

		Female LPS	1	5	11	15	32
	%	Male Saline	46.9	40.6	9.4	3.1	100.0
		Male LPS	40.6	43.8	6.3	9.4	100.0
		Female Saline	16.1	9.7	45.2	29.0	100.0
		Female LPS	3.1	15.6	34.4	46.9	100.0

Validation of discriminant functions for Experiment 2, for cases grouped by gavage treatment and sex, by original case classification and leave-one-out cross validation. 64.6% of the original grouped cases are correctly classified by the discriminant functions. In the leave-one-out cross-validation test, the discriminant functions are recalculated excluding one case, and all cases are recalculated. This algorithm is repeated for the exclusion of each case. In the leave-one-out test, 45.7% of cross-validated grouped cases were correctly classified.

Table 4.8 Supplemental Table 5. Original classification and cross-validation of discriminant functions for Experiment 1.

			Predicted Group Membership				
		SexByNaloxone	Male Saline	Male (+)-naloxone	Female Saline	Female (+)-naloxone	Total
Original	Count	Male Saline	20	10	2	0	32
		Male (+)-naloxone	9	22	1	0	32
		Female Saline	2	3	20	7	32

		Female (+)-naloxone	2	1	7	21	31
	%	Male Saline	62.5	31.3	6.3	.0	100.0
		Male (+)-naloxone	28.1	68.8	3.1	.0	100.0
		Female Saline	6.3	9.4	62.5	21.9	100.0
		Female (+)-naloxone	6.5	3.2	22.6	67.7	100.0
Cross-validated	Count	Male Saline	18	11	3	0	32
		Male (+)-naloxone	15	14	3	0	32
		Female Saline	5	3	14	10	32
		Female (+)-naloxone	3	2	11	15	31
	%	Male Saline	56.3	34.4	9.4	.0	100.0
		Male (+)-naloxone	46.9	43.8	9.4	.0	100.0
		Female Saline	15.6	9.4	43.8	31.3	100.0
		Female (+)-naloxone	9.7	6.5	35.5	48.4	100.0

Validation of discriminant functions for Experiment 2, for cases grouped by (+)-naloxone treatment and sex, by original case classification and leave-one-out cross validation. 65.4% of the original grouped cases are correctly classified by the discriminant functions. In the leave-one-out cross-validation test, the discriminant functions are recalculated excluding one case, and all cases are recalculated. This algorithm is

repeated for the exclusion of each case. In the leave-one-out test, 48.0% of cross-validated grouped cases were correctly classified.

Table 4.9 Supplemental Table 6. Meta analysis of behavioral outcomes for WT males not treated with TLR4 antagonists (n=15/group).

Measure	Mean - Exp 1	UL - Exp 1	LL - Exp 1
Time in Center Zone (sec)	-35.279	-49.205	-8.662
Time Spent in Stereotypic Circling (sec)	-7.507	-14.94	-0.075
Stereotypic Counts	-122.29	-248.4	3.827
Ambulatory Counts	181.43	-366.53	729.388
Ambulatory Episodes	5.572	-29.281	40.424
Ambulatory Time (sec)	16.914	-18.596	52.425
Ambulatory Distance (cm)	235.66	-489.47	960.787
Resting Time (sec)	-6.764	-42.879	29.351
Avg Velocity (cm/sec)	-1.462	-4.527	1.603
Zone Entries	38.121	19.832	56.411
Time in Stretch Posture (sec)	-0.093	-0.391	0.206
Jump Counts	-11.143	-39.197	16.911
Jump Time (sec)	-2.65	-8.241	2.941
Clockwise Reversals	-0.429	-5.304	4.447
Counter-Clockwise Reversals	-0.143	-4.901	4.615
	Mean - Exp 2	UL - Exp 2	LL - Exp 2
Time in Center Zone (sec)	-12.075	-50.105	25.955
Time Spent in Stereotypic Circling (sec)	-8.112	-18.754	2.529

Stereotypic Counts	-173	-355.79	9.787
Ambulatory Counts	-219.13	-536.6	98.354
Ambulatory Episodes	-39.5	-105.7	26.705
Ambulatory Time (sec)	-14.338	-37.113	8.438
Ambulatory Distance (cm)	-346.34	-804.57	111.894
Resting Time (sec)	26.231	-5.972	58.434
Avg Velocity (cm/sec)	0.24	-4.106	3.626
Zone Entries	-17.5	-30.366	-4.635
Time in Stretch Posture (sec)	-0.019	-0.139	0.102
Jump Counts	-53.25	-109.91	3.407
Jump Time (sec)	-7.564	-17.68	2.555
Clockwise Reversals	-3	-13.19	7.19
Counter-Clockwise Reversals	3	-9.357	15.357
	Mean - Meta Analysis	UL - Meta Analysis	LL - Meta Analysis
Time in Center Zone (sec)	-7.507	-14.94	-0.075
Time Spent in Stereotypic Circling (sec)	-7.701	-13.211	-2.192
Stereotypic Counts	-138.29	-232.14	-44.447
Ambulatory Counts	-70.45	-449.73	308.832
Ambulatory Episodes	-9.233	-50.721	32.256
Ambulatory Time (sec)	-1.157	-31.406	29.091
Ambulatory Distance (cm)	-111.28	-670.99	448.426
Resting Time (sec)	10.481	-21.821	42.782
Avg Velocity (cm/sec)	-0.999	-3.173	1.174
Zone Entries	10.001	-44.504	64.505
Time in Stretch Posture (sec)	-0.029	-0.131	0.072

Jump Counts	-25.879	-65.241	13.484
Jump Time (sec)	-3.772	-8.19	0.646
Clockwise Reversals	-0.895	-4.863	3.072
Counter-Clockwise Reversals	0.252	-3.75	4.254

95% confidence interval values for Experiment 1, Experiment 2, and the meta analysis. UL=Upper Limit. LL=Lower Limit.

Table 4.10 Supplemental Table 7. Meta analysis of behavioral outcomes for WT males not treated with TLR4 antagonists (n=15/group).

Measure	Mean - Exp 1	UL - Exp 1	LL - Exp 1
Time in Center Zone (sec)	-35.279	-49.205	-8.662
Time Spent in Stereotypic Circling (sec)	-7.507	-14.94	-0.075
Stereotypic Counts	-122.29	-248.4	3.827
Ambulatory Counts	181.43	-366.53	729.388
Ambulatory Episodes	5.572	-29.281	40.424
Ambulatory Time (sec)	16.914	-18.596	52.425
Ambulatory Distance (cm)	235.66	-489.47	960.787
Resting Time (sec)	-6.764	-42.879	29.351
Avg Velocity (cm/sec)	-1.462	-4.527	1.603
Zone Entries	38.121	19.832	56.411
Time in Stretch Posture (sec)	-0.093	-0.391	0.206
Jump Counts	-11.143	-39.197	16.911
Jump Time (sec)	-2.65	-8.241	2.941
Clockwise Reversals	-0.429	-5.304	4.447

Counter-Clockwise Reversals	-0.143	-4.901	4.615
	Mean - Exp 2	UL - Exp 2	LL - Exp 2
Time in Center Zone (sec)	-12.075	-50.105	25.955
Time Spent in Stereotypic Circling (sec)	-8.112	-18.754	2.529
Stereotypic Counts	-173	-355.79	9.787
Ambulatory Counts	-219.13	-536.6	98.354
Ambulatory Episodes	-39.5	-105.7	26.705
Ambulatory Time (sec)	-14.338	-37.113	8.438
Ambulatory Distance (cm)	-346.34	-804.57	111.894
Resting Time (sec)	26.231	-5.972	58.434
Avg Velocity (cm/sec)	0.24	-4.106	3.626
Zone Entries	-17.5	-30.366	-4.635
Time in Stretch Posture (sec)	-0.019	-0.139	0.102
Jump Counts	-53.25	-109.91	3.407
Jump Time (sec)	-7.564	-17.68	2.555
Clockwise Reversals	-3	-13.19	7.19
Counter-Clockwise Reversals	3	-9.357	15.357
	Mean - Meta Analysis	UL - Meta Analysis	LL - Meta Analysis
Time in Center Zone (sec)	-7.507	-14.94	-0.075
Time Spent in Stereotypic Circling (sec)	-7.701	-13.211	-2.192
Stereotypic Counts	-138.29	-232.14	-44.447
Ambulatory Counts	-70.45	-449.73	308.832
Ambulatory Episodes	-9.233	-50.721	32.256
Ambulatory Time (sec)	-1.157	-31.406	29.091

Ambulatory Distance (cm)	-111.28	-670.99	448.426
Resting Time (sec)	10.481	-21.821	42.782
Avg Velocity (cm/sec)	-0.999	-3.173	1.174
Zone Entries	10.001	-44.504	64.505
Time in Stretch Posture (sec)	-0.029	-0.131	0.072
Jump Counts	-25.879	-65.241	13.484
Jump Time (sec)	-3.772	-8.19	0.646
Clockwise Reversals	-0.895	-4.863	3.072
Counter-Clockwise Reversals	0.252	-3.75	4.254

Legend: 95% confidence interval values for Experiment 1, Experiment 2, and the meta analysis. UL=Upper Limit. LL=Lower Limit.

5 DISCUSSION

The three projects discussed in this dissertation all converge to highlight the potential impact of gut microbiota on the function of the gut barrier, which is the first direct interface the gut microbiota has with the host. Gut barrier function modulates several key mechanisms via which the microbiota can influence brain function and behavior, including, for example, type and level of local immune activity, alteration of systemic short-chain fatty acid levels and afferent vagal activity, all of which have been linked to modifying behavior (Heyman, 2005; Marietta et al., 2018; Spielman et al., 2018). Within this discussion, I will outline how gut microbiota may have influenced gut barrier function across these three projects. These studies highlight the need for a broader methodological approach to gut microbiota analysis that will provide more investigative traction in the future, which may put less emphasis on gut microbiota analysis and more on identifying core functional consequences of “dysbiosis”.

5.1 Core Factors Influence Gut Microbiota In Differing Conditions: LPS and Gut Barrier Dysfunction as Recurring Themes

Chapter 2 focused on identifying differences in microbiota in Brattleboro (*Avp-/-*) and Long Evans (WT) rats, which differ in behavior (Brattleboro rats are less social and less anxious than their WT Long Evans counterparts (Feifel et al., 2009; Balazsfi et al., 2015; Paul et al., 2016)). Most researchers attribute this to the absence of systemically and centrally-released vasopressin, which affects hydration status, influences the immune system, and acts as a neuromodulator in the CNS. All of these can affect behavior. We sought to identify potential alterations in gut microbiota in Brattleboro rats

that may also influence their behavioral status. For this, we needed an analysis that could identify genera and species that may have been altered. Prior work by our collaborators Chassaing and colleagues used QIIME and a machine-learning algorithm called nearest-shrunken centroid to identify broad changes in gut microbiota and to identify which specific microbial species best represent each of the designated groups, respectively in identifying changes in microbiota that might explain various effects of gut dysfunction on physiology (e.g., (Chassaing et al., 2015)). QIIME is able to identify population-wide differences in microbial samples between groups, using an unbiased principle components analysis approach. However, while this platform is able to determine phylum-level differences in gut microbiota, it is not designed to investigate genus- and species-level differences between groups. For this, they used the nearest shrunken centroid classification approach, which is a machine-learning approach that identifies the microbial species that best characterize sample groups (Choi et al., 2017). However, this statistical approach does not present information on the size of the abundance disparity between groups for species identified to be differentially abundant (Dabney, 2005). We needed to identify a more rigorous approach to identifying differentiated taxa. For this, we used LEfSe (Linear Discriminant Analysis coupled with Effect Size)--a tool developed to address this specific problem of identifying and ranking which microbial taxa associate most closely with each sample group. Other tools used to identify marker genes and biomarkers have been adapted for the purposes of gut microbiota analysis, including DESeq and EdgeR, however, a comparison of these tools revealed that LefSe was one of the most conservative in identifying differentiated taxa

(Paulson et al., 2013). This was important as we wanted to identify the few microbial taxa that most robustly associate with each treatment condition.

The most salient differentiated taxa across the various AVP genotypes was that of the genus *Lactobacillus* between male WT, heterozygous, and homozygous *Avp* knockout genotypes, being most abundant in KO rats and least abundant in WT rats. This may be a significant change as *Lactobacilli* improve multiple aspects of gut barrier protection, which may impact baseline behavioral profile of the host. Certain *Lactobacillus* strains have been reported to increase the expression of anti-inflammatory cytokine IL-10 in innate immune cells and to counteract TLR4 cell signaling pathways in vitro (Villena and Kitazawa, 2014). *Lactobacilli* have also been shown to upregulate the expression of intestinal epithelial tight junction protein, thereby increasing gut barrier protection against gut luminal microbiota (Anderson et al., 2010; Karczewski et al., 2010). This has been replicated in vivo, as two species of Lactobacillus have also been shown to protect against an experimental model of necrotizing enterocolitis by upregulating tight junction protein expression (Blackwood et al., 2017). In addition to increasing intestinal epithelial tight junction protein levels and upregulating anti-inflammatory cytokine expression, *Lactobacilli* also increase immunoglobulin A (IgA) levels (Kim et al., 2016). IgA is an antibody predominantly produced in the lamina propria that binds to gut luminal microbiota along the mucus lining of the gut, reducing its entry in the lamina propria. Decreasing the magnitude and immunological impact of gut microbiota that cross the intestinal epithelial barrier is a key function of *Lactobacilli* and differing levels of this crucial gut microbe may impact baseline levels of gut

inflammation that may impact a wide assortment of body systems, including the brain and host behavior.

While Lactobacillus was not found to be significantly different across genotypes in female rats, a couple other bacterial taxa linked with immune status were also found to be differentiated across the females. *Lachnospira spp.* were found to be most abundant in heterozygous rats and *Holdemania spp.* were most abundant in WT rats. As discussed in Chapter 2, both of these taxa are associated with increased inflammation. In addition, they have both been associated with conditions of increased gut permeability. Emerging connections between gut barrier dysfunction, systemic LPS burden, and kidney dysfunction have implicated Lachnospira as one of the most salient pro-inflammatory bacterial taxa to drive chronic kidney disease (Lun et al., 2018; Meijers et al., 2018). However, a clearer link has emerged for *Holdemania*: Abundance of *Holdemania spp.* decrease upon alcohol withdrawal, when alcohol-induced increases in gut permeability abate (Leclercq et al., 2014). Thus, while changes in gut microbiota across genotypes may be sex-specific, changes observed across both sexes converge on taxa that may influence gut barrier function and baseline states of systemic inflammation. Future studies could be directed to identifying which aspects of gut barrier function are altered and the functional consequences of such alterations.

In Chapter 3, we asked whether adding emulsifiers to the diet, which induces changes in the microbiota and thereby systemic inflammation, also influences behavior. To obtain a more robust view on the effects of two differing emulsifier treatments (carboxymethylcellulose [CMC] and polysorbate-80 [P80]) on behavior in male and female mice, we used multivariate analysis. Gut microbiota have cumulative and

pleitropic effects on host physiology and behavior. While it is important to identify robust effects on individual measures, it is also informative to investigate the effects of gut microbial manipulations on behavioral syndromes, and more broadly, syndromic effects across host physiology, brain function, and behavioral output. This provides insight into a more holistic understanding of the effects of gut microbiota on the host. We adopted the approach of utilizing multiple discriminant analysis (MDA) to identify significantly cumulative effects of gut microbiota on the host. MDA identifies features that contribute to significant differences between groups across multiple dimensions. **Figure 5.1** illustrates the basic principles of MDA across two dimensions. Herein, there are two separate sample populations, represented by ellipses A and B, demonstrated across two dimensions—axis x and axis y. Along axis x, the sample groups nearly completely overlap, sharing over 50% overlap. Along axis y, the sample groups completely overlap. However, it is clear that, when taken across both dimensions, the sample groups represent two separate populations. One useful method of conceptualizing the brain's own use of such a high-dimensional approach to classification is with facial recognition. When looking at individual features such as eye color, hair color, nose size, etc., it may be very difficult to differentiate between individual faces. However, the subtle differences in the combination of all of these features combine to help classify a face to a specific person.

Likewise, we used multiple discriminant analysis to reveal interesting sex-specific effects of emulsifier treatment on behavioral and physiological syndromes. While the two emulsifier treatments largely converge to produce similar behavioral changes in subject mice, namely altered anxiety-related and social behavior, they have unique

effects on gut microbiota composition that may affect unique aspects of anxiety behavior. For example, while anxiety behavior is increased by both P80 and CMC, the collective syndrome of anxiety-related behaviors (thigmotaxis, rearing, stereotypic circling, etc.) is affected differently by the two emulsifiers. This may be due to differing strengths in the bond formation properties of these emulsifiers. P80 is a nonionic surfactant which inhibits biofilm formation of *P aeruginosa* and other gram-negative and gram-positive bacterial species (Toutain-Kidd et al., 2009). Sodium carboxymethyl cellulose is an anionic surfactant, which, like sodium dodecyl sulfate, may predominantly affect gram-positive bacteria (Galbraith et al., 1971; Walton et al., 2008; Toutain-Kidd et al., 2009). Indeed, as shown in Chapter 3, the two emulsifiers uniquely impact sex differences in abundance of certain subsets of microbial taxa. CMC treatment roughly maintained the number of taxa that are sexually differentiated, and P80 treatment appeared to greatly reduce the number of sexually differentiated taxa. Also, the two emulsifier treatments uniquely impacted sex differences in behavior relative to that observed in WT mice, where P80 largely maintained these sex differences while CMC appeared to reverse sex differences across a certain subset of anxiety-like and repetitive behaviors. Therefore, the lower number of sexually differentiated taxa induced by P80 treatment may be important for maintaining sex differences in behavior. Investigation of the sexually differential microbiota induced by CMC treatment may yield additional clues to understanding the effects of gut microbiota on sexually differentiated behavior.

Emulsifier treatment had been shown to erode the mucus lining, thereby allowing the luminal gut microbiota to encroach upon the intestinal epithelial lining (Chassaing et

al., 2015). While the two emulsifiers may uniquely impact the specific compositional changes in gut microbiota observed in male and female mice, they both converge to increase systemic burdern of LPS in both sexes (Chassaing et al., 2015). Increased antibodies against LPS were observed in the serum and increased levels of LPS was also detected in the feces. This may be the result of either increased abundance of gram-negative bacteria or increased shedding of LPS by gut-resident gram-negative bacteria. LPS may be a key player by way of its ability to increase intestinal permeability and gut immune activation. The differing consortia of gut microbiota induced by each emulsifier treatment in each sex may elicit, for example, unique immune effects and uniquely stimulate the vagus nerve in such a manner to elicit differing effects on behavior, thus providing a potential hypothesis for how increased LPS observed across treatments with differing effects can elicit unique outcomes.

The studies conducted in Chapter 3 suggest that a shift to a more gram-negative microbiota profile resulting from both CMC and P80 treatment (or at least increased shedding of LPS by the gut microbiota) may be responsible for the metabolic and behavioral changes observed in emulsifier-fed mice. I tested the plausibility of LPS as a core gut-derived factor influencing anxiety-related behavior in the gut-derived endotoxin study discussed in Chapter 4. Oral administration of LPS models acute changes in gut microbiota that may be observed in various conditions exhibiting gut dysbiosis, such as the emulsifier-induced increases in serum and fecal LPS levels. We found that oral administration of LPS increased anxiety-related behaviors in subject mice, and that this depended on systemic TLR4 expression as *Tlr4-/-* mice were protected from the effects of LPS treatment. While LPS increased anxiety-related behaviors in both male and

female mice, it may have done so via sexually different TLR4 signaling mechanisms, as an antagonist that blocks the TRIF pathway in TLR4, (+)-naloxone, independently reversed sex differences for a unique subset of locomotor and anxiety behaviors relative to those observed in untreated mice.

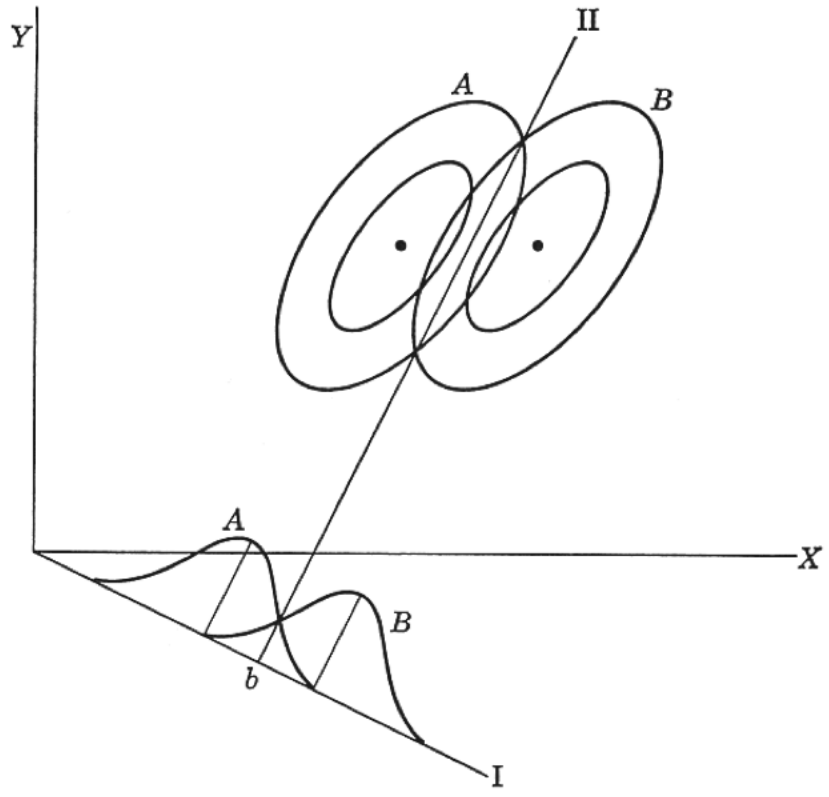

Figure 5.1 Multiple discriminant analysis (MDA) identifies syndromic effects across multiple dimensions

Two separate sample population, denoted as ellipses A and B, which represent two separate populations, demonstrated across two dimensions—axis x and axis y. Along axis x, the sample groups nearly completely overlap, sharing approximately 50% overlap. Along axis y, the sample groups completely overlap. However, it is clear that,

when taken across both dimensions, the sample groups represent two separate populations.

5.2 The Gut Inflammation-Brain Connection

As mentioned in the Introduction, one of the mechanisms whereby TLR4-induced gut inflammation may affect brain activity is via immune signaling transmitted through the vagus nerve. Vagus nerve activity directly influences brain transcriptomes with behavioral implications for psychiatic disorders such as schizophrenia (Klarer et al., 2018), and resection of the vagus nerve (vagotomy) is often used to demonstrate the central importance of vagal nerve communication to changes in the brain and behavioral output (Forsythe et al., 2014). Relevant to anxiety-related disorders, vagotomy prevented increases in anxiety behavior induced by DSS colitis (Bercik et al., 2011b). In addition, germ-free mice also demonstrate changes in brain neurotransmitter and receptor levels that may be driven by changes in vagal tone (Diaz Heijtz et al., 2011). The vagus nerve has a rich network of innervations within the lamina propria (Powley et al., 2011), expresses innate immune receptors (Goehler et al., 1997), and is critical to certain aspects of sickness behavior response induced by intraperitoneal injections of LPS or IL-1 (Bluthe et al., 1996). Further work on how the vagus nerve responds to gut-derived inflammation, what signals are conveyed, and how these signals specifically affect CNS function and behavioral output is still needed.

Barrier defenses maintain the distance between commensal gut microbiota and the lamina propria, which houses vagus nerve efferents. One important component of this defense system involves a wide selection of innate immune receptors expressed on

the intestinal epithelial cells. One such immune receptor is toll-like receptor 5 (TLR5). Deletion of TLR5 from intestinal epithelial cells ($TLR5^{\Delta IEC}$) alters the composition of gut microbiota in such a manner that allows for the growth of microbes typically targeted by TLR5 activation, particularly bacteria bearing the inflammagen “flagellin”-the protein building block of flagellum, a whip-like appendage that facilitates bacterial locomotion (Chassaing et al., 2014a). This compositional change, which can be classified as “dysbiosis”, facilitates thinning of the mucus layer and encroachment of gut microbiota toward the intestinal epithelium, provoking more robust activation of intestinal epithelial innate immune receptors which promotes gut inflammation. Just as emulsifiers promote recomposition of gut microbiota, gut inflammation, and various aspects of metabolic syndrome (increased adiposity, impairments in glucose control), so does intestinal deletion of TLR5 (Chassaing et al., 2014a). TLR5 also has an impact on LPS, as increased levels of fecal LPS are observed in $TLR5^{\Delta IEC}$ mice (Chassaing et al., 2014a). Preliminary data from my colleague Nicole Peters suggest that global TLR5 deletion impacts social and anxiety behavior, and future investigation of the behavioral effects of TLR5 deletion from intestinal epithelial cells is warranted. Outside of identifying broad-scale effects on gut inflammation, identifying core consequences of gut microbial changes observed in $TLR5^{\Delta IEC}$ provides a handle on mechanisms that may drive the physiological and behavioral effects observed in TLR5-KO mice. Here, again, although it is unlikely to be the sole component driving the physiological effects observed in $TLR5^{\Delta IEC}$ mice, overgrowth of pathogenic gram-negative bacteria resulting in shedding excess LPS and contributing to metabolic endotoxemia, may be important factors contributing to metabolic and behavioral dysregulation found in TLR5-KO mice.

With respect to gut microbiota and gut physiology, while the effects of gut bacteria on TLR4 have received a lot of attention, and the importance of TLR5 in recognizing bacterial flagellin as an important regulator of gut barrier integrity is gaining recognition, different TLRs expressed by intestinal epithelial cells recognize other microbial kingdoms and cross-talk with other non-TLR innate immune receptors that contribute to gut homeostasis. TLR4 not only recognizes LPS but is also activated by fungal mannan oligosaccharides, a component of their cell wall, and glycoinositol phospholipids produced by certain parasites (Kawai and Akira, 2011). As discussed in Chapter 4, TLR4 has two separate signaling cascades, and while LPS may predominantly activate the Myd88 pathway, mannan activation of TLR4 predominantly activates the TRIF pathway (Netea et al., 2005). While TLR4-KO mice do not exhibit signs of intestinal inflammation, they do exhibit alterations in gut microbiota that likely spill over to non-bacterial microbial kingdoms such as alterations in fungal composition and load (Perez-Pardo et al., 2018). Activation of the two TLR4 pathways and activation of other TLRs by LPS may yield downstream effects on cytokine profile and subsequent vagal and CNS activation unique to a specific microbiota composition. Indeed, this may explain the opposing effects of LPS on behavior in WT versus *Tlr4-/-* mice discussed in Chapter 4.

Other innate immune receptors also recognize important components of gut microbiota. For example, certain subsets of immune receptors, including TLR3, TLR5, TLR9, and some of the NOD-like receptors, evolved to recognize viruses, as they respond to RNA and DNA sequestered in the endoplasmic reticulum of damaged and infected cells engulfed by epithelial and immune cells (Kawai and Akira, 2011).

However, receptors such as TLR2 and dectin-1, evolved to recognize conserved components of fungi (Kawai and Akira, 2011). There is also a division of labor in the recognition of gram-positive and gram-negative bacteria, with TLR4 recognizing LPS on gram-negative bacteria and TLR2 recognizing cell wall components present on gram-positive bacteria (Takeuchi et al., 1999). Activation of each individual receptor triggers a unique inflammatory cascade that has unique effects on the host, some broadly “anti-inflammatory” and some “pro-inflammatory” (Kawai and Akira, 2011). Also, through cross-talk of induced cytokines and receptor oligomerization, receptors interact with each other to propagate specific anti-microbial responses. How these various subsets of the immune system affect the adult CNS and specifically vagal efferents is unknown, as very little work to-date has characterized immune receptor signaling and proximal and distal vagus nerve activation by various non-TLR4 gut innate immune receptor stimulation. As was shown in Chapter 4, LPS produced identifiable behavioral effects in TLR4-KO mice, suggesting that LPS activated other innate immune receptors, such as TLR2, that signal to the vagus nerve and CNS. Which specific immune receptors are responsible and what those signals are will be the focus of future investigation.

5.3 Non-bacterial Microbial Components Affect Gut Barrier and Brain Function

While gut bacteria strongly influence host physiology and behavior, there are other components of gut microbiota that may signal along the gut-brain axis, including viruses, fungi, archaea and parasites. Species across all of these microbial kingdoms activate a wide variety of innate immune receptors that may have unique effects on the vagus nerve and the CNS. Future investigation of the gut-brain axis will need to

broaden investigation into these other kingdoms. For example, are there significant differences in the gut fungal communities between WT and Brattleboro rats that may also contribute to behavioral differences between the two strains? Differences in viral, parasite, and archaeal communities may also contribute to differences in metabolites, vagal signaling, and immune activation that all signal back to the CNS.

Bacteria serve as a reservoir for a specific type of viruses, namely bacteriophage (Fischetti, 2005). One means by which viruses may affect CNS function and behavior is through their ability to modulate the gut bacterial landscape. The mucus layer of the intestinal lining is especially enriched in bacteriophages, which contributes to barrier defense against commensal bacteria (Barr et al., 2013). A bacteriophage can infect and kill their bacterial hosts but can also serve to transmit genetic information between bacterial hosts (Columpsi et al., 2016). In this way, bacteriophages may either serve to maintain homeostasis by preventing overgrowth of certain species and also may help to maintain biological diversity within bacterial species (Columpsi et al., 2016). In addition to serving as defense against mucosal encroachment of gut bacteria, bacteriophages also function as antiviral defense mechanisms against host-infecting viruses by producing proteins that block cell entry or upregulate interferon gamma production (Miedzybrodzki et al., 2005). Bacteriophages are not the only viruses present in gut bacteria. There are also viruses that infect fungi, archaea, and eukaryotic host cells. Some of these viruses may modulate the gut barrier, such as adenoviruses, which may trigger Celiac disease (Kahrs et al., 2019). Additionally, while bacteriophages are mostly touted for their protective capacity, they can also infect human host cells and may be a causative agent in some diseases (Tetz and Tetz, 2018). Likewise, groups of viruses

known to traditionally infect human cells are studied as disease agents, little is known regarding the possible existence of beneficial commensal human-infecting viruses that may also be transmitted by the gut microbiome. Such viruses may be transmitted directly to the brain, and unpublished data presented at a recent Society for Neuroscience conference suggests that the presence or absence of gut microbiota (that is, comparing conventionally-colonized and germ-free mice) may influence the presence of viruses and bacteria in the brain. To date, little is known about the composition of the gut virome, as there does not exist a conserved biomarker between viruses similar to 16S rRNA in bacteria (Columpsi et al., 2016). However, advances in metagenomics are beginning to facilitate basic surveys into gut viral composition.

Another component of the gut microbiome is the fungus kingdom, comprising the gut “mycobiome”. Again, to date, relatively little attention has been paid to the gut mycobiome, let alone to its effects on the brain. While there are studies on the effects of exogenous mold exposure on brain function and behavior (Crago et al., 2003), what remains to be explored are the typical endogenous fungal compositional profile (which may include both commensal and pathogenic fungi) and its specific effects on the host. While they account for a relatively small portion of the gut microbiome, they may still exert a powerful impact on host physiology and behavior. For example, *Candida albicans*, just as any opportunistic pathogen, accounts for a very small percentage of the total biomass of the gut microbiota, but still has important consequences for the immune system, CNS, and host behavior (Underhill and Iliev, 2014; Neville et al., 2015). There are challenges with sequencing this community. One salient point is that while conserved evolutionary biomarkers (barcodes) exist to discern fungal species (namely

conserved fragments of 18S and 28S rRNA, as well as internal transcribed spacer regions [ITS1 and ITS2]), reference databases are far less extensive than those developed for bacterial communities. Some of these barcodes also do not allow discrimination beyond the genus level (Nilsson et al., 2019). Again, this community does not exist in isolation, and has dynamic links both across fungal species and between fungi and bacteria which may affect the bacterial landscape and have downstream signaling effects on the CNS (Witherden et al., 2017). Independent of its effects on bacteria, fungi also activate innate immune receptors that promote type-2 inflammation (dectin-2, TLR2, etc.), which may have effects on brain function and behavior distinct from those induced by LPS and gram-negative bacteria (Kawai and Akira, 2011). One example is the potential opposing effects of LPS-induced inflammation and type-2 inflammation on repetitive and compulsive-like behavior.

Fungi and bacteria may have opposing effects on behavior given their opposing effects on inflammation. “Type 1” inflammation, or the canonical inflammatory response (which includes the response to injected LPS), and “type 2” inflammation, or the allergic immune response produce cytokines that counteract each other—type 1 inflammation typically downregulates type 2 inflammation, and vice versa (Kidd, 2003). As the sickness response involving type 1 inflammation includes a “sickness behavior” response, which consists of lethargy, social withdrawal, and increases in anxiety behavior (Dantzer, 2009), type-2 inflammation may trigger a different behavioral response, including increases in repetitive behavior and compulsive-like grooming designed to limit tic, fungal, parasite exposure (Hart, 1994; Reber et al., 2011). For example, mouse strains that mount robust type 1 immune responses exhibit lower

levels of repetitive behavior than strains that mount type 2 immune responses (Thomas et al., 2009). Behavioral differences between mouse strains exhibiting differing immune biases may also be transmitted via fecal microbiota transfer (Bercik et al., 2011a). Widening the scope beyond the bacterial component of gut microbiota may help address some of the mysteries surrounding the behavioral effects of gut microbiota transfer. Most gut microbiota transfer procedures are not performed anaerobically, and a large majority of transferred fecal species are dead upon transfer to the new host (Chu et al., 2017). Gut fungal species which are predominantly facultative anaerobes, however, survive. Fungi may also impact bacterial signaling to the host through influencing the gut barrier—some fungal components, such as zymosan, downregulate intestinal tight junction protein expression (Li et al., 2015b), which may increase metabolic endotoxemia and associated downstream effects on host physiology and behavior. However, in this situation, fungal-induced biases in inflammation may alter the response to LPS, promoting an enhanced type-2 response (perhaps by enhancing the TLR4-TRIF pathway over TLR4-Myd88). Again, this may have contributed to the opposing effects of orally-administered LPS in WT and *Tlr4-/-* mice described in Chapter 4.

5.4 Collapsing Complexity by Assessing the Functional Impact of Microbiota

Complexity within gut microbiota is not limited to the impact of less studied kingdoms, but also to the extensive functional redundancy across highly divergent microbial populations. An example of this complexity lies in variations of gut “dysbiosis” that have been shown to transmit metabolic syndrome. First, as described in Chapter 3,

two separate food emulsifiers that induce separate and very sex-specific alterations in the gut microbiome both increase body weight and other signs of metabolic syndrome in subject mice. Second, fecal microbiota collected from a genetic model of obesity, leptin-deficient *ob/ob* mice, was the first example that showed that obesity can be transmitted via fecal microbiota transfer (Ley et al., 2005; Turnbaugh et al., 2006). Third, later studies using microbiota from high-fat diet fed mice showed similar results (Turnbaugh et al., 2008). Comparing gut microbiota across all three models will undoubtedly yield very distinct microbial population clusters. Attempts to identify individual taxa associated with the three separate obesity models may show interesting candidates but will likely not be present in all subjects. An example of this is the difficulty of some researchers to replicate the Firmicutes-to-Bacteriodetes ratio originally observed in obese mice and humans (Ley et al., 2005). Subsequent work either found no changes in this ratio (Duncan et al., 2008; Million et al., 2013; Rosenbaum et al., 2015) or an inverse of this ratio in obese subjects (Schwiertz et al., 2010; Ignacio et al., 2016), along with more fine-grained increases and decreases of species within both the *Firmicutes* and *Bacteroidetes* phyla within obese subjects (Bruce-Keller et al., 2015; Jung et al., 2018). Nevertheless, later work also confirmed the finding of an increased *Firmicutes*-to-*Bacteroidetes* ratio in some obese subjects, suggesting that this compositional change may serve as a significant factor in a subset of the disease (Koliada et al., 2017). Nevertheless, transfer of microbiota from all of these models of obesity into metabolically healthy mice induces metabolic syndrome in the subject (Turnbaugh et al., 2006; Turnbaugh et al., 2008; Chassaing et al., 2015).

Investigation of core functional effects of varied dysbiotic changes observed within a disorder, as opposed to attempting to identify and isolate individual culprits within the gut microbiome, will provide a critical window into therapeutic targets. As highlighted in this Discussion, one potential core mechanism may be increases in gut permeability, which can be triggered by a wide assortment of bacteria, viruses, and fungi. For example, both clinical subjects and rodent models of autism exhibit increases in intestinal permeability, alterations in gut microbiota, and increases in anxiety-related behaviors (Hsiao et al., 2013; Fiorentino et al., 2016). Decreases in Prevotella are noted as constituting one of the most robust microbial taxa changes associated with autistic subjects (Kang et al., 2013; Kang et al., 2018). However, Prevotella is observed to be increased in the maternal immune activation model of autism (Hsiao et al., 2013). However, this model also exhibits increased intestinal permeability (Hsiao et al., 2013). Even when there are consistent trends across studies, this may not reveal the whole story. Parkinson's disease is another condition where the contributions of gut microbiota are becoming more apparent (Sampson et al., 2016). Here, too, gut barrier dysfunction is also associated with disease progression (Houser and Tansey, 2017). Overgrowth of Proteobacteria in Parkinson's subjects is consistently noted across studies (Forsyth et al., 2011; Keshavarzian et al., 2015; Scheperjans et al., 2015; Unger et al., 2016; Li et al., 2017; Qian et al., 2018). Increases in Proteobacteria are also associated with autism (Williams et al., 2012; Wang et al., 2013) and obesity (Cani et al., 2007). As mentioned earlier, Proteobacteria can increase intestinal permeability (Jakobsson et al., 2015), primarily mediated through activation of innate immune receptor TLR4 by its cell surface antigen lipopolysaccharide (Guo et al., 2015). However, here too, noting increases

in Proteobacteria is insufficient to infer functional consequences, as even different strains of *Escherichia coli*, a species in the Proteobacteria phylum, carry lipopolysaccharide molecules with differing levels of immunogenicity, with some serving as TLR4 agonists and others as TLR4 antagonists (Coats et al., 2005). Current high-throughput gut microbiota sequencing efforts, which identify bacteria by a portion of its 16S rRNA signature, cannot distinguish between strains, and some sequence tags fail to discriminate beyond the genus or family level (Fukuda et al., 2016). The complexities imposed by gut microbiota analysis demands an investigative focus on core mechanisms and consolidated functional consequences.

A specific etiologic trigger, such as gut barrier dysfunction, which may be precipitated by several different changes in bacterial composition, likely interacts with several environmental and genetic risk factors to precipitate specific disease outcomes. This model is highlighted in **Figure 5.2**. Increases in gut barrier dysfunction may alter other gut microbial communication pathways to the brain, which may include modifying systemic short-chain fatty acid levels and afferent vagal activity. For example, both rare mutations and ingestion of environmental toxins have been suggested to contribute to disease onset in PD patients, perhaps both triggering gut barrier dysfunction that increases host exposure to gut microbiota-derived prions that trigger α-synuclein misfolding along the vagus nerve (Smith and Parr-Brownlie, 2018; Zeng et al., 2018).

Sex differences in various host systems, such as the immune system (Klein and Flanagan, 2016), may combine with dysbiotic changes in gut microbiota to exacerbate disease outcomes in one or the other sex. For example, bacterial antigens may activate differing types of inflammation in males and females (e.g., generate a more pro-allergic

immune profile in males) (Kelly and Gangur, 2009), which promote differing types of neuroinflammation with potentially different behavioral effects. On the other hand, my study described in Chapter 4 revealed that a common microbial antigen elicited similar behavioral but differential immune responses in males and females. Only a few studies have directly investigated how gut microbiota and host sex factors interact. For example, one landmark study by (Markle et al., 2013) identified robust sex differences in gut microbiota in adult mice and revealed that gut microbiota from males when transferred to females may elevate testosterone levels in females (Markle et al., 2013). The recent National Institutes of Health mandate to include both female and male subjects in biomedical research should undoubtedly be applied to the study of gut microbiota, which is likely to reveal many more sex-specific effects of gut microbiota on the host.

Gut barrier dysfunction is not the only gut microbiota-associated factor that has context-specific effects. Short chain fatty acids have been associated with both proinflammatory and anti-inflammatory effects, based on host context (Kuo et al., 2014; Zhang et al., 2016). These are not likely the only gut microbiota-associated factors that affect host biology differently based on context. Furthermore, these gut microbial effects are likely to both converge and cancel each other out, so identifying dominant factors within each disease state will be key to identifying prominent mechanisms of action.

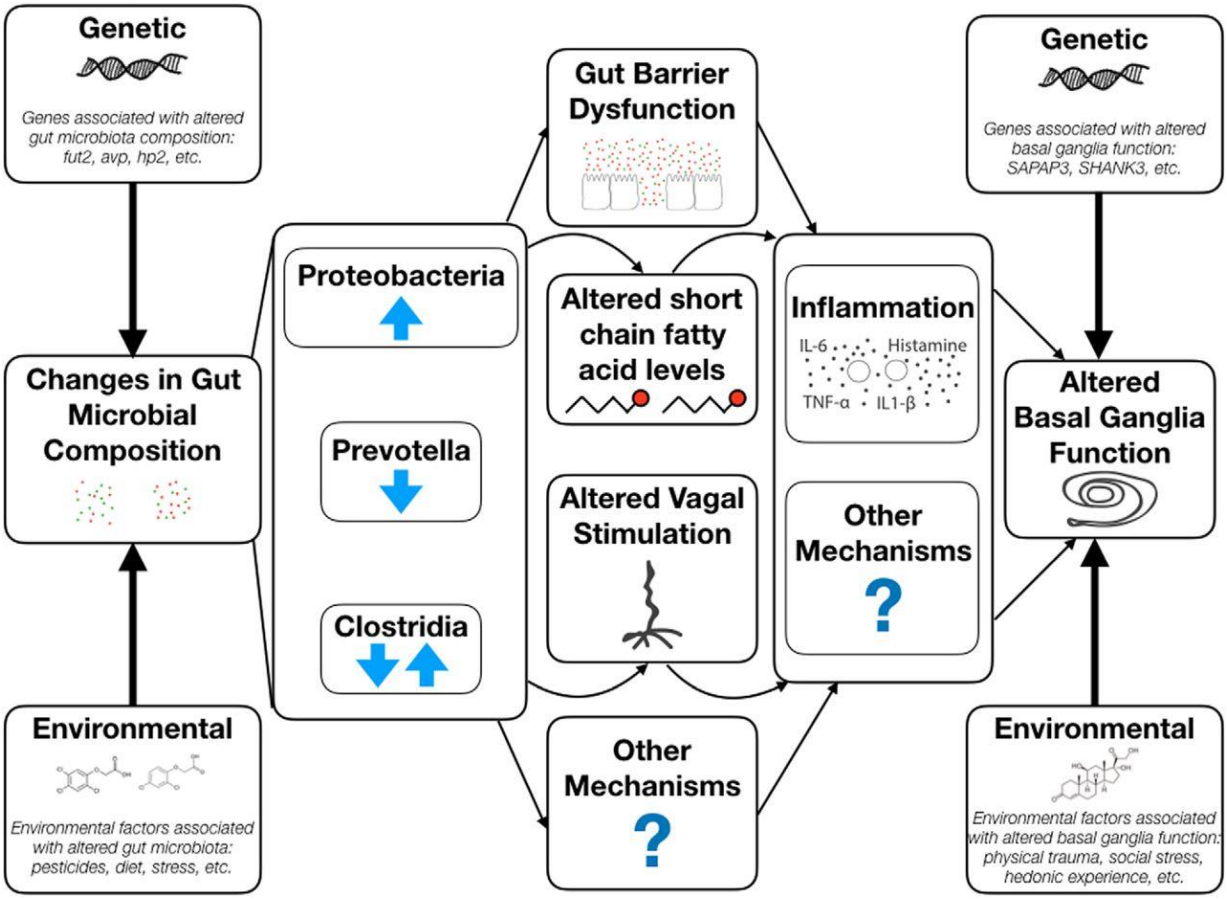

Figure 5.2 Genetic and environmental factors interact with gut microbiota to induce specific effects on the host.

Gut microbial alterations occur within the context of genetic and environmental factors that shape basal ganglia-associated disease susceptibility. These host factors affect both gut microbial composition and basal ganglia function. Common microbial alterations associated with increased disease risk include increases in Proteobacteria, decreases in Prevotella, and alterations in Clostridia, which are all associated with increased gut barrier dysfunction. Other risk factors, such as altered short-chain fatty acid levels, increased vagal activation, and other mechanisms (e.g., the release of other bacterial metabolites) may also result from gut microbial alteration. Increased systemic inflammation and neuro-inflammation are common endpoints of all of these alterations,

but other gut-to-brain mechanisms also contribute to basal ganglia disease etiology. Ultimately, gut-derived factors that alter basal ganglia function interact with other preexisting genetic and environmental susceptibility factors to shape specific disease outcomes. Copied with permission from Fields et al. (2018) Defining dysbiosis in disorders of movement and motivation. J Neurosci.

5.5 Utilizing Multidimensional Analyses to Explore Complex Systems

Multidimensional analyses reveal emergent patterns within biological data not available from analyzing individual measures, reflecting the multidimensional nature of these complex systems. Here, we use multidimensional analyses to explore compositional patterns of bacterial populations within gut microbiota and to explore emergent behavioral syndromes across treatment groups. An example of the requirement for dimensional approaches to analysis is exemplified in the various levels of analysis pursued in biological disciplines such as neuroscience, where patterns of activity observed in individual neurons are best contextualized within larger patterns of chemical and electrical activity within and across brain regions. For example, neuronal firing during the encoding or retrieval of memory events may appear stochastic when observed in the individual neuron, but recognizable patterns of activity emerge when brain activity is measured at the regional or whole brain level (Johnson et al., 2009).

When multidimensionality is taken into consideration, the robustness of biological systems is often revealed. Complex systems can be redundant and systems that appear similar can have widely diverging functions/effects. This point was demonstrated in Chapter 3, where gut microbiota populations were compared for emulsifier treatments

across two different studies. As demonstrated by LefSe, the treatments elicited differing population compositions across the two studies, but emulsifiers impacted the subjects' metabolic state in a similar fashion across studies. In Chapter 2, we used LefSe to identify core taxa that are associated with the Brattleboro rat genotype. Here, we found a select few taxa that correlated with genotype, but other compositional changes in microbial community structure may also result from the observed genotypic changes. Dimensions such as circadian fluctuations in microbial populations, microbe-to-microbe interactions, strain differences across bacteria, and environmental subniches that alter the functional output of the same bacterial strain are also unexplored. Additionally, other kingdoms that constitute the gut microflora, such as fungi, viruses, parasites, and archaea, are not surveyed by 16S rRNA biomarker sequencing, which only enumerates bacterial populations. These other kingdoms elicit categorically differing immune responses, and likely have unique effects on other host systems as well. As a result, there are entire categories of interactions missing from most modern microbiome analyses, which will also likely exhibit time, strain, and micro-environment specific complexity. As a wider array of dimensions are captured of gut microbiota and their effects on the host CNS, important and unintuitive system dynamics will likely emerge that may help define core principles of gut microbiota to brain signaling.

While multivariate analyses are routinely used in psychology, most prominently in survey studies, this approach remains under-utilized in preclinical rodent behavior analyses. In a survey, individual questions are meant to capture an aspect of a larger construct. In rodent studies, individual tests are designed to stand alone as measures of a particular psychological attribute (e.g. anxiety), demonstrating face validity (apparent

similarity to human emotional construct) and predictive validity (clinically validated psychiatric drugs have predicted effects on animal subjects) (Chadman et al., 2009). However, there are a number of tests that are said to measure the same psychological attribute, but these tests are often inconsistent, varying between treatments, subjects, and trials. Other confounds such as prior experience, housing conditions, and environment contribute to inconsistencies in these data (Chadman et al., 2009; Silverman et al., 2010; Kazdoba et al., 2016). However, multivariate approaches to behavioral analysis may provide a means to compare similar behavioral syndromic effects across study cohorts. This was demonstrated in Chapter 4 where LPS treatment elicited significantly different effects in separate groups of anxiety-like behavior between study cohorts (Experiment 1 vs Experiment 2), but multivariate analysis demonstrated that LPS induced an anxiety-related behavioral syndrome across both cohorts. Comparing results across one or two primary measures from only one test, as is traditionally done with rodent behavioral studies, may yield similar inconsistencies from cohort to cohort that may be standardized by multivariate analysis.

From vagal stimulation to systemic breach of gut-derived toxins and from stimulation of systemic inflammation to systemic release of bacterial metabolic byproducts, such as short chain fatty acids, these mechanisms of action can be driven by multiple compositional profiles and can have differential effects based on host biology. Future studies will need to further identify not only compositional differences in gut microbiota associated with health and disease, but also the context-specific functional effects of these microbial alterations. This will serve as a critical step toward developing psychiatric treatments leveraging gut microbial manipulation.

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