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Identifying the Role of Vasopressin and Oxytocin in the Microbiota-Gut-Brain-Behavior Axis

Nicole Peters

IDENTIFYING THE ROLE OF VASOPRESSIN AND OXYTOCIN IN THE MICROBIOTA-GUT-BRAIN-BEHAVIOR AXIS

by

NICOLE PETERS

Under the Direction of Geert de Vries, PhD

ABSTRACT

The gut microbiota is a complex ecosystem of microorganisms that form a bidirectional communication pathway with the brain, called the gut-brain axis. In addition to their roles in mediating host metabolism and digestion, a wealth of research is identifying roles for the gut microbiota in neural development and function, immune modulation, and behavioral expression. Many neural targets of gut-brain axis signaling have been identified, but little attention has been paid to vasopressin and oxytocin. Vasopressin and oxytocin are neuropeptides that are targets of immune signaling and are implicated in the control of anxiety-like, depressive-like, and social behaviors, making them likely mediators in the communication between the gut and the brain. As

the immune system is a main signaling pathway in the gut-brain axis, it is possible that vasopressin and oxytocin would be affected through immune system activation to result in behavioral alterations seen in microbiota dysbiosis. To test these predictions, we used pro-inflammatory and anti-inflammatory microbiota manipulation mouse models to identify the roles of vasopressin and oxytocin in the gut-brain axis. First, we demonstrated that microbiota is needed for proper vasopressin and oxytocin system development by using a germ-free mouse model. Second, we explored the impacts that chronic intestinal inflammation has on behavior and neuropeptide expression in Toll-like receptor 5 knockout (T5KO) mice. Third, we investigated whether the behavioral phenotype in T5KO mice is microbiota dependent. Collectively, these experiments provide support to the hypothesis that microbiota alter the vasopressin and oxytocin systems through an immune-mediated pathway to alter the behavior of both mouse models. They also support the use of T5KO mice in investigating the interplay between chronic, low-grade inflammation and psychiatric disorders. Future experiments are needed to uncover the exact mechanisms underlying the microbiota-gut-brain-behavior axis and understanding this axis will provide a basis for developing microbiota-based therapeutics to treat CNS disorders.

INDEX WORDS: TLR5, Germ-free mice, Gut-brain axis, Gut microbiota, Vasopressin, Oxytocin, Behavior

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NICOLE PETERS

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NICOLE PETERS

Committee Chair: Geert de Vries

Committee: Nancy Forger

Aras Petrulis

Andrew Gewirtz

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

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DEDICATION

To my grandfather, Daniel Krzesinski, and my mother, Victoria Peters, for showing me that a Ph.D. is achievable, and always placing an emphasis on education.

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LIST OF ABBREVIATIONS

5-HT- 5-hydroxytryptamine

ABC- avidin-biotin complex

AH- anterior hypothalamus

ANOVA- analysis of variance

ASD- autism spectrum disorders

AVP- arginine vasopressin

BBB- blood-brain barrier

BDNF- Brain derived neurotrophic factor

BNST- bed nucleus of the stria terminalis

BNSTmv- medial ventral bed nucleus of the stria terminalis

CC- conventionally colonized

CEC- cerebral endothelial cells

CNS- central nervous system

CRF- corticotrophin-releasing factor

CVO- circumventricular organs

DAB- nickel-enhanced diaminobenzidine

DMH- dorsomedial nucleus of the hypothalamus

EEC- enteroendocrine cells

EPM- elevated plus maze

EZM- elevated zero maze

GABA- gamma-aminobutyric acid

GF- Germ-free

HPA- hypothalamus-pituitary-adrenal axis

Iba-1- ionized calcium binding adapter molecule 1

ICV- intracerebroventricular

IEC- intestinal epithelial cells

IHC- immunohistochemistry

IL-1β- interleukin-1β

ir- immunoreactivity

L/D Box- light/dark box

Lcn-2- lipocalin-2

LHb- lateral habenula

LPS- lipopolysaccharide

LS- lateral septum

LSV- ventral lateral septum

MANOVA- multivariate analysis of variance

MD- mediodorsal nucleus of the thalamus

MHC- major histocompatibility complex

mRNA- messenger ribonucleic acid

NF-κB- Nuclear Factor kappa-light-chain-enhancer of activated B cells

NGS- normal goat serum

OFT- open field test

OXT- oxytocin

PBS- phosphate-buffered saline

PVN- paraventricular nucleus of the hypothalamus

PVT- paraventricular nucleus of the thalamus

RE- recolonized

SCFA- short-chain fatty acids

SCN- suprachiasmatic nucleus

SEM- standard error of the mean

SON- supraoptic nucleus

SPZ- subparaventricular zone

T5KO- Toll-like receptor 5 knockout

T5KO-g- mice treated with Toll-like receptor 5 knockout microbiota

TBS- Tris-buffered saline

TCT- three chamber sociability test

TLR- Toll-like receptor

TLR4- Toll-like receptor 4

TLR5- Toll-like receptor 5

TNF-α- tumor necrosis factor alpha

TST- tail suspension test

WT- Wild-type

WT-g- mice treated with wild-type microbiota

1 INTRODUCTION

1.1 Microbiota-Gut-Brain Axis

Mammals and other animals are inhabited by millions of microorganisms on any surface that is exposed to the outside environment, including the skin, mouth, gut, and vaginal canal (Backhed et al., 2005). These microorganisms, called the microbiota, consist of bacteria, fungi, parasites, and other microorganisms, and are estimated to equal or outnumber by up to ten times the host's cells (Sender et al., 2016). Bacteria comprise by far the largest portion of the microbiota and typically form a symbiotic relationship with the host (Chow et al., 2010). The microbiota is a complex ecosystem and perturbations to the ecosystem can result in the proliferation of non-beneficial species, leading to a state of dysbiosis (Rojo et al., 2017). While the definition of dysbiosis is generally unclear (reviewed in Fields et al., 2018), one can consider it to be a shift in the composition such that there is a pro-inflammatory effect on the body. Dysbiosis has been shown to be a component of a number of disorders, such as inflammatory bowel disease and psychiatric disorders (Carding et al., 2015).

While the microbiota is present throughout the body, the role of the gut microbiota has been particularly well-studied with regards to its relation to human health, as it plays roles in host digestion, metabolism, and even diet selection (Rezzi et al., 2007; Ley et al., 2008; Alcock et al., 2014; Andoh, 2016; Gentile and Weir, 2018). However, the gut microbiota has functions that extend past the intestines, achieved through numerous communication pathways with the rest of the body. For example, the microbiota can interact directly with the nervous system through activation of the vagus nerve or through the enteric nervous system (Yoo and Mazmanian, 2017). Gut

microbiota can produce metabolic byproducts such as short-chain fatty acids that signal the cells of the intestinal epithelium, or they can also produce neurotransmitters, such as serotonin, that can communicate with the rest of the body (Aidy et al., 2015; Morrison and Preston, 2016; Kennedy et al., 2017). Finally, they can directly influence the immune system by stimulating immune cells to release pro- or anti-inflammatory cytokines, either locally or systemically, or by recruiting and activating immune cells in the gut or brain (Chassaing & Gewirtz, 2016; Fiebiger et al., 2016; Mcdermott & Huffnagle, 2014). Signaling through this route is the main focus of investigation throughout this dissertation.

Through these pathways, the gut microbiota can communicate with the brain to change behavior, as evidenced by their role in psychiatric disorders. In fact, a wealth of research has been performed in the past 15 years on the effects changing the composition of the microbiota has on the brain and behavior. One of the primary models used is germ-free (GF) mice. GF mice, raised in sterile isolators, have a number of physiological and behavioral changes from conventionally colonized (CC) mice. For example, they have ceca that are twice as large as normally colonized mice due to their inability to adequately digest fiber (Wostmann and Bruckner-Kardoss, 1959; Respondek et al., 2013). They also have decreased anxiety-like behavior, decreased sociability, and cognitive impairments (Clarke et al., 2013; Desbonnet et al., 2014; Neufeld et al., 2011). GF mice are a useful model for identifying neural systems affected by the microbiota, due to the severity of a global knockout of microbiota (Luczynski et al., 2016). Furthermore, GF mice are excellent for identifying critical periods of microbiota influence on brain development, because experimenters can

recolonize them at specific developmental time points. In fact, a number of studies have used this manipulation to identify temporal effects of microbiota on brain development and behavior (Diaz Heijtz et al., 2011; Erny et al., 2015; Lu et al., 2018; Neufeld et al., 2011). In addition, GF mice are useful as an anti-inflammatory physiological system, due to their immature immune systems and lack of immune challenges from the environment (Abrams et al., 1963; Clarke et al., 2013). Despite the fact that GF mice are not an ethologically relevant model, they are an excellent way to identify neural systems affected by microbiota.

There are a number of models that use different manipulations to mimic intestinal inflammation. One way that is used frequently in the literature is to administer lipopolysaccharide (LPS), the component of the membrane of Gram-negative bacteria, either intraperitoneally or by oral gavage to result in a proxy of bacterial infection (Fields et al., 2018; Hug et al., 2018; Taylor et al., 2012). Another way is to increase the inflammatory nature of the microbiota through introduction of pro-inflammatory bacterial species, such as *Campylobacter jejuni* or *Escheria coli* (Chassaing et al., 2014; Lyte et al., 1998). Alternatively, there are genetic manipulations that result in chronic, low-grade intestinal inflammation. One such manipulation is the use of Toll-like receptor 5 knockouts.

Organisms use pattern recognition receptors to identify invading pathogens by recognizing conserved bacterial, fungal, or viral components on the pathogens in the body, and once activated, they begin a signaling cascade to promote an inflammatory response to rid the body of the pathogen (Takeuchi and Akira, 2010). One such family of pattern recognition receptors are the toll-like receptors (TLRs); different TLRs each

recognize a different component (Rakoff-Nahoum et al., 2004; Yiu et al., 2016). For example, TLR4 recognizes LPS and TLR5 recognizes flagellin, a component of the flagella of motile bacteria (Chow et al., 1999; Hayashi et al., 2001). TLR5 is most frequently located on the basolateral surface of the intestinal epithelial layer, indicating that bacteria need to pass through the epithelium to activate these receptors (Gewirtz et al., 2001). In TLR5 knockout (T5KO) mice, TLR5 receptors are not present to catch any invading bacteria, giving the invading bacteria longer to reproduce and resulting in a more intense immune response once detected (Vijay-Kumar et al., 2008). Over time, these immune challenges build to form a phenotype characterized by increased inflammation, glucose sensitivity, insulin insensitivity, increased triglycerides, and obesity, all characteristics of intestinal inflammation and metabolic syndrome (Vijay-Kumar et al., 2007; Vijay-Kumar et al., 2010).

Unlike many of the previously-discussed models that increase the inflammatory state of the gut, the physiological changes of the T5KO mouse model depend on the gut microbiota. When GF wild-type (WT) mice are colonized with microbiota from T5KO mice, they develop the symptoms of metabolic syndrome seen in the T5KO mice (Vijay-Kumar et al., 2010). This is due to increased levels of *Proteobacteria* in the T5KO mice as well as an increased bacterial load (Carvalho et al., 2012). In addition, the mucus layer that protects the intestinal epithelium from contact with the microbiota is also thinner in these mice, which allows bacteria to be closer and more adherent to the intestinal wall (Carvalho et al., 2012). Unsurprisingly, the physiological phenotype of T5KO mice is due to the loss of TLR5 in the intestinal epithelial cells (IEC) (Chassaing et al., 2014a). IEC-specific T5KO recapitulates the physiological phenotype of the

whole-body TLR5 deficiency. T5KO mice are an excellent model to investigate the microbiota-gut-brain-behavior axis because they show microbiota-dependent chronic inflammation and their physiological changes are well characterized by our collaborators. Furthermore, unlike GF mice, T5KO mice are relevant to human health. While humans with a 75% reduction in TLR5 function do not exhibit the same phenotype as our T5KO mice (Gewirtz et al., 2006), the phenotype of these mice is reminiscent of metabolic syndrome, which is increasingly plaguing Western society (Vijay-Kumar et al., 2010).

Metabolic syndrome comprises a constellation of symptoms, including obesity, dyslipidemia, glucose intolerance, and hypertension, which increases the risk for cardiovascular disease and type 2 diabetes. It is estimated that 20-25% of the adult population has metabolic syndrome, making it a significant health concern (Mazidi et al., 2016). Multiple studies have demonstrated an association between anxiety-like and depressive-like behaviors and metabolic syndrome in mice, rats, and humans (Dinel et al., 2011; de Cossío et al., 2017; Rebolledo-Solleiro et al., 2017; Penninx and Lange, 2018a). A similar pattern is seen in the comorbidity between functional gastrointestinal disorders like irritable bowel syndrome and psychiatric disorders (De Palma et al., 2014; Midenfjord et al., 2019; Zamani et al., 2019), underscoring the importance of understanding the factors that cause this association. The TLR5 knockout mouse, with its phenotype resembling functional gastrointestinal disorders and metabolic syndrome, is an excellent choice for examining the gut-brain axis.

1.2 Microbiota and Neuropeptides

There has been an explosion of research into identifying and understanding where and how gut microbiota manipulations affect neural circuitry, and a number of neurotransmitters have been implicated in this pathway, including serotonin, corticotropin-releasing hormone (CRF), brain-derived neurotrophic factor (BDNF), glutamate, and dopamine, among others (Baj et al., 2019; Bercik et al., 2011; Crumeyrolle-Arias et al., 2014; Guida et al., 2018a; Liu et al., 2016; Lukíc et al., 2019; Nishino et al., 2013; O'Leary et al., 2018; O'Mahony et al., 2015; Palomo-Buitrago et al., 2019; Singhal et al., 2019). Despite their roles in many behaviors affected by microbiota, including social, anxiety-like and depressive-like behaviors, little is known about the roles that the neuropeptides vasopressin and oxytocin play in the gut-brain axis (reviewed in Caldwell et al., 2008; Jurek & Neumann, 2018; Kormos & Gaszner, 2013; Neumann & Landgraf, 2012). Vasopressin and oxytocin both increase social behaviors but play opposite roles in anxiety-like behaviors (Neumann and Landgraf, 2012). Vasopressin has an anxiogenic effect, evidenced by increased central vasopressin mRNA in rats bred for high anxiety-like behavior, and reduced anxiety-like behavior in vasopressin receptor knockout mice (Bielsky et al., 2004; Wigger et al., 2004). Oxytocin is anxiolytic, shown by increased anxiety-like behavior in oxytocin knockout mice and reductions in anxiety-like behavior when oxytocin is administered centrally (Amico et al., 2004; Ring et al., 2006). Similar patterns are seen in the moderation of depressive-like behavior by vasopressin and oxytocin (Arletti and Bertolini, 1987; Keck et al., 2003; Ring et al., 2010). Furthermore, these systems are

sensitive to peripheral and immune signals, making these neuropeptides a likely target in the gut-brain axis (Nava et al., 2000).

To date, very few studies have investigated the interaction between microbiota and vasopressin and oxytocin in the brain, and these studies are generally restricted to mRNA expression in the hypothalamus. For example, Desbonnet and colleagues found that antibiotic treatment beginning at weaning reduced vasopressin and oxytocin mRNA in the hypothalamus in adulthood (Desbonnet et al., 2015), but they did not see any changes in vasopressin mRNA after treatment with the probiotic *Bifidobacteria* in rats (Desbonnet et al., 2008). They also found that in a maternal separation paradigm, there was no effect of the probiotic *Bifidobacterium infantis* administration on vasopressin mRNA in the amygdaloid cortex or the hypothalamus (Desbonnet et al., 2010). This same research group found that NIH Swiss mice showed a decrease in vasopressin receptor 1a mRNA expression in the hypothalamus in a maternal immune activation model that was associated with increased intestinal permeability and motility (Morais et al., 2018). Furthermore, our lab found that rats with a naturally-occurring knockout of vasopressin show a sex-specific shift in gut microbiota composition that is correlated with anxiety-like behavior (Fields et al., 2018b). While these studies point to a role of vasopressin in response to microbiota manipulations, or vice versa in the case of Fields et al. (2018b), they are restricted only to the hypothalamus and mRNA expression. More detailed analysis is required to truly understand the role that vasopressin plays in the gut-brain axis.

A series of elegant mechanistic experiments demonstrated that the probiotic *Lactobacillus reuteri* alleviates social deficits in multiple mouse models of autism

spectrum disorders (ASDs) by increasing oxytocin expression in the paraventricular nucleus of the hypothalamus (PVN; Buffington et al., 2016; Sgritta et al., 2019). This suggests that oxytocinergic signaling is affected by the actions of bacteria, and it is possible that behavioral alterations from changes to the gut microbiota are occurring by disrupting the oxytocin system. In addition, stressed mice treated with antibiotics from weaning had reduced oxytocin mRNA in the hypothalamus, and prenatal stress reduced oxytocin receptor mRNA in the cortex and altered the gut microbiota (Desbonnet et al., 2015; Gur et al., 2019), suggesting an interaction between stress, microbiota and oxytocin expression. Another study did not find any change in oxytocin expression in antibiotic-treated rats, which may point to species-specific effects of microbiota on oxytocin (Kentner et al., 2018). Finally, human studies found that higher levels of circulating oxytocin is associated with increased *Dialister* genera, associated with glucose metabolism, but no correlation between plasma oxytocin and composition of the fecal microbiota was found in ASD patients (Tomova et al., 2015; Barengolts et al., 2018). While more is known about oxytocin's place in the gut-brain axis than that of vasopressin, it is worthwhile to investigate it further for the potential therapeutic implications of oxytocin.

1.3 Summary of Chapters

The studies in this dissertation explore the microbiota-gut-brain axis in the context of the effect of microbiota on behavior. While many studies recently have explored this axis, there is still a vast deficiency in our knowledge on how microbiota composition affects the body at the levels of microbiota ecosystem, gut physiology, gut to brain communication pathways, the brain, and behavior. A primary deficiency is in

the roles vasopressin and oxytocin play in the gut-brain axis. We hypothesize that microbiota change social, anxiety-like and depressive-like behaviors in part by affecting neuropeptide pathways implicated in those behaviors, namely vasopressin and oxytocin. We investigate this through the use of an anti-inflammatory model, GF mice, that show decreases in anxiety-like behaviors, and a pro-inflammatory model, T5KO mice, that should show increases to anxiety-like behaviors. We expand the findings that microbiota influence anxiety-like and social behaviors by investigating the role that the neuropeptides oxytocin and vasopressin may play in this pathway and correlating those roles with behavioral expression.

In Chapter 2, I used an anti-inflammatory mouse model, GF mice, to investigate if the gut microbiota is necessary for proper development of the vasopressin and oxytocin systems. GF mice show myriad behavioral abnormalities, including reduced anxiety-like behavior and decreased sociability, but the mechanisms underlying these behavioral changes are still not fully defined. We hypothesized that oxytocin and vasopressin are involved in modulating behavior in response to signals from the microbiota, because these neuropeptides are sensitive to peripheral immune signals, and they are involved in the expression of anxiety-related and social behavior (Chikanza and Grossman, 2002; Caldwell et al., 2008b; Li et al., 2017b; Jurek and Neumann, 2018b). Thus, we characterized vasopressin and oxytocin immunoreactivity in weanling and adult mice in the production sites (paraventricular nucleus of the hypothalamus, supraoptic nucleus, suprachiasmatic nucleus), and projection sites of these neuropeptides (Rood and De Vries, 2011; Rood et al., 2013). We were also interested in whether changes in these neuropeptides in GF mice could be rescued by colonization with conventional

microbiota at weaning. We settled on this time point because puberty seems to be a critical period in the effects of microbiota on brain development (Markle et al., 2013). Finally, despite the well-characterized behavior of adult GF mice, less is known about their behavioral development. Thus, we investigated anxiety-like and social behaviors in weanling-aged GF mice. Overall, we found that the lack of microbiota affects vasopressin immunoreactivity in weanling-aged animals but has no effect in the adults, whereas oxytocin is increased both at weaning and in adulthood in GF mice. These changes to the vasopressin and oxytocin systems are associated with behavioral alterations. Furthermore, recolonization at weaning is not sufficient to recapitulate normal vasopressin and oxytocin expression, which suggests that microbiota is needed for proper neuropeptide system development.

In Chapter 3, we used a pro-inflammatory mouse model to explore whether chronic intestinal inflammation (1) affects anxiety-like and social behaviors, (2) is associated with changes to the oxytocin and vasopressin systems, and (3) whether these changes are due to microbiota changes. The use of inflammatory agents in microbiota or behavioral research is not new. A primary manipulation used is administration of LPS, which activates TLR4 and is responsible for inducing sickness behavior. However, we were interested in what effect chronic intestinal inflammation, similar to what would occur in disorders like inflammatory bowel syndrome, has on neuropeptides and behavior. We chose to use a T5KO model that has been well phenotyped by our collaborators and that has microbiota-dependent symptoms of chronic intestinal inflammation and metabolic syndrome. First, we behaviorally phenotyped T5KO mice in a variety of anxiety-like, depressive-like and social behavior

tests. Next, we examined vasopressin and oxytocin immunoreactivity in brain regions that receive signals from the periphery and are involved in mediating these behaviors. Finally, we used T5KO microbiota transplantation into GF mice to determine if the microbiota is sufficient to cause the T5KO behavioral phenotype. We found that T5KO mice are characterized by increased anxiety-like behavior and reduced locomotion that is correlated with increased vasopressin immunoreactivity, and that this behavioral phenotype is not induced by T5KO microbiota transplant into GF mice.

Combined these studies point to the need for future investigation into vasopressin as a mediator between microbiota composition changes and behavioral expression, as well as introduce a model of intestinal inflammation that should be utilized in gut-brain axis research. More broadly, they point to the need for more mechanistic or pathway driven studies to uncover the effects that microbiota have on the central nervous system (CNS) in both health and disease states. In Chapter 4, I discuss the larger context for the results of my experiments in the microbiota-gut-brain-behavior axis as well as discuss the future directions of my research.

2 MICROBIOTA ARE NECESSARY FOR PROPER NEURAL VASOPRESSIN AND OXYTOCIN DEVELOPMENT IN MICE

Nicole V. Peters, Mary K. Holder, Daniel Teuscher, Grace Signiski, Matthew J. Paul, Jack Whylings, Andrew T. Gewirtz, Benoit Chassaing and Geert J. de Vries

2.1 Abstract

Gut microbiota can influence anxiety-like, depressive-like and social behaviors, but the underlying mechanisms are still mostly unknown. Because vasopressin (AVP) and oxytocin (OXT) play significant roles in the control of these behaviors, we investigated whether being raised in a germ-free (GF) environment permanently alters AVP and OXT circuits. We found that compared to conventionally colonized (CC) mice, adult GF mice had region- and sex-specific alteration of AVP and OXT immunoreactivity, and these effects were not rescued by recolonization of GF mice at weaning. There was also region- and sex-specific changes to microglia, a marker of neuroinflammation and measured by Iba-1 immunoreactivity and cell number, in AVP and OXT-expressing nuclei of GF mice. Since AVP and OXT influence juvenile anxiety-like and social behaviors, this led us to investigate whether the behavioral and neural phenotype of GF mice is present at weaning. We found that weanling-aged GF mice show decreased anxiety-like behavior and decreased social behavior, similar to adult GF mice, as well as changes to AVP and OXT immunoreactivity. These results suggest that AVP, OXT, and microglia are influenced by microbiota during development, and the changes to these systems may contribute to the altered behavioral phenotype of GF mice.

2.2 Introduction

The microbiota that colonizes our gut, skin, oral cavity, and other regions of the body exposed to the external environment affects the physiology of the body as well as the brain (Foster and McVey Neufeld, 2013; Mayer et al., 2015; Dinan and Cryan, 2017). Germ-free (GF) mice, which are born and raised in sterile isolators, have been widely used to identify systems affected by microbiota (reviewed in Cryan & Dinan, 2012; Luczynski et al., 2016). Adult GF mice have a well-established behavioral and physiological profile, characterized by decreased anxiety-like, depressive-like, and social behaviors, particularly in less stress-responsive mouse strains (Borre et al., 2014; Desbonnet et al., 2014; Farzi, Fröhlich, & Holzer, 2018), as well as immature immune system development and low intestinal inflammation (Foster and McVey Neufeld, 2013; Luczynski et al., 2016). Colonizing GF mice before puberty with conventional microbiota restores behavior to normal levels in GF mice (Desbonnet et al., 2014; Diaz Heijtz et al., 2011; Pan et al., 2019a), however, colonizing after puberty does not (Sudo et al., 2004). This suggests a critical period for the effects of microbiota on behavior, and thus on the underlying neural circuitry.

It is still unclear what neural circuitry is affected by the low inflammatory status of GF mice to change their behavior. Others have shown that monoamines, including noradrenaline, dopamine, and serotonin, as well as brain-derived neurotrophic factor, and corticotropin-releasing factor are affected in the brains of GF mice (Guida et al., 2018b; Baj et al., 2019; Lukić et al., 2019; Palomo-Buitrago et al., 2019; Pan et al., 2019b; Singhal et al., 2019). However, relatively little attention has been paid to the neuropeptides AVP and OXT, despite their major roles in anxiety, depression, and

social behavior (reviewed in Bredewold & Veenema, 2018; Caldwell, 2017; Jurek & Neumann, 2018). Previous experiments show that antibiotic treatment reduced AVP and OXT mRNA expression in the hypothalamus and altered anxiety-like and social behaviors (Desbonnet et al., 2015). Furthermore, OXT is needed during probiotic treatment to ameliorate social behavior impairments in a maternal high fat diet model (Buffington et al., 2016; Sgritta et al., 2019). These results point to the need to further investigate how the microbiota affects these neuropeptides.

As gut inflammation can cause neuroinflammation (Rizzetto et al., 2018; Serra et al., 2019), we used microglia, the macrophages of the central nervous system, as a marker of neuroinflammation (Colonna and Butovsky, 2017). GF mice tend to have an immature microglia profile, including increased microglial number, disturbed neural surveillance parameters, and diminished response to pathogens (Erny et al., 2015; Castillo-Ruiz et al., 2018; Thion et al., 2018), and recolonization with microbiota before puberty restores the microglia to a more mature profile.

In the present study, we examined the immunoreactivity of AVP, OXT, and microglia in adult GF and conventionally colonized (CC) mice, and in GF mice colonized with microbiota at weaning (recolonized; RE) in brain regions implicated in the control of social and anxiety-like behaviors. We found that OXT immunoreactivity was increased in GF mice in some regions, whereas there was no difference in AVP immunoreactivity between GF and CC mice. We also found site- and sex-specific effects of lack of microbiota to Iba-1 (a marker of microglia) immunoreactivity and Iba-1 positive cell count. Recolonization did not rescue immunoreactivity to the levels of CC mice in any of the three systems we examined, but rather increased AVP immunoreactivity.

This discovery led us to question whether the deficits in AVP, OXT, and microglia were already present in weanling-aged mice. To do this, we first established that weanling-aged mice show the same GF behavioral phenotype as described in adults, defined by decreased anxiety-like behavior and social behavior. Then, we characterized AVP, OXT and Iba-1 immunoreactivity in the same regions as the previous experiment to determine if these systems are altered by weaning from the lack of microbiota in early life, and if changes to these systems may explain the changes in behavior in GF mice.

2.3 Materials and Methods

2.3.1 Animals

Swiss-Webster mice (GF, CC, and RE) were obtained from our breeding program at Georgia State University. All non-sterile mice (CC and RE) were housed in ventilated transparent Optimouse cages (35.6 x 48.5 x 21.8cm) lined with Bed-O-Cobs® bedding, with nestlets and shelters for enrichment. Animals were kept on a 12h:12h light:dark cycle (lights off at 1900 EST) and ambient temperature was kept at 23°C. Food (Purina rodent chow no. 5001) and water were available *ad libitum*. Animals were weaned at postnatal day 21 (P21) and housed with littermates of the same sex and genotype. All procedures were in accordance with the Guide for Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the Georgia State University.

Germ-free mice were maintained in a Park Bioservices isolator as previously described (Chassaing et al., 2015) and allowed *ad libitum* access to autoclaved food (Purina rodent chow no. 5001) and water. Adult and weanling-aged mice were obtained

from the established GF breeding colony at Georgia State University. Recolonized mice were removed from the isolator at P21 and orally administered with 200uL of fecal suspension from a sex-matched donor, then kept in conventional animal housing as described above.

Weanling CC Swiss Webster mice used for behavioral testing were obtained from Taconic (Germantown, NY) and allowed to habituate to the animal facility before use in the behavioral experiment, and weanling GF mice were obtained as described above. None of the animals used in behavioral testing were used for the anatomical experiments.

2.3.2 Behavioral Testing

Weanling-aged mice (P21) were tested in the social interaction, marble burying, and elevated plus maze tests, in that order, after removal from the isolators or animal facility and an hour-long habituation to the testing room. The tests were ordered this way, from least to most anxiogenic, to reduce residual stress from the previous test (Mcilwain et al., 2001). GF and CC mice were not tested on the same day to reduce the possibility of contamination of the GF mice. Behavioral testing began 3 hours after the beginning of the light phase of the light:dark cycle, with overhead lights as illumination, and was completed within a 6-hour time frame in one day to minimize microbiota colonization. Mice were immediately moved from the social interaction arena to the marble burying arena, then were returned to their home cage for between 30 minutes to 3 hours between the marble burying and EPM. This variation in time was due to the animal order being randomized for each test. Apparatuses were cleaned with 70% ethanol (between animals) or Vimoba solution (between treatment groups and at the

start and end of each testing day; chlorine dioxide; Quip Laboratories, Wilmington, DE) to remove the scent of the previous mouse. An experimenter blind to treatment conditions scored all behavioral tests.

2.3.2.1 Social Interaction

A Plexiglas arena (24cm W X 46 cm L) was filled with 2 cm of Alpha-dri bedding (Shepherd Specialty Paper, Fibercore, Cleveland, OH, USA). Two mice from the same litter (and therefore the same treatment) were placed into the arena and video recorded for 10 minutes. Time spent walking, immobile, grooming, allogrooming, rearing, digging, and investigating the other mouse were scored using Observer XT 11.5 (Noldus Information Technology, Wageningen, The Netherlands).

2.3.2.2 Marble Burying Test

A Plexiglas arena (24cm W X 46 cm L) was filled with 4 cm of Alpha-dri bedding (Shepherd Specialty Paper, Fibercore, Cleveland, OH, USA). Mice were placed into the arena for a 5-minute habituation period, then removed in order to place 20 marbles (17mm) in an evenly spaced, 4x5 grid on top of the bedding. Mice were returned to the center of the arena and their behavior was video-recorded for 10 minutes. The number of marbles buried during this period, defined as being half or more covered by bedding, the latency to bury the first marble, and total time spent digging were quantified using Observer XT 11.5 (Noldus Information Technology, Wageningen, The Netherlands).

2.3.2.3 Elevated Plus Maze

A standard mouse elevated plus maze (EPM) was used, with 2 open arms and 2 closed arms. The arms were 10 cm W x 50 cm L, connected by a 10 cm X 10 cm center square. Closed arms had a wall height of 40 cm, and the maze was elevated 50

cm from the floor. At the beginning of the test, mice were placed in the center square of the arena and allowed to freely explore for 5 min. Video trials were recorded from a digital camera mounted above the apparatus that was connected to a computer. The number of entries into the open and the closed arms of the apparatus, time spent in open and closed arms, and total distance traveled were quantified by AnyMaze version 4.96 (Stoelting, Co., Wood Dale, IL).

2.3.3 Euthanasia and Tissue Collections

After completion of behavioral testing, mice were deeply anesthetized using isoflurane (5%v/v). Blood was collected by retrobulbar intraorbital capillary plexus. Hemolysis-free serum was collected by centrifugation of blood using serum-separator tubes (Becton Dickinson, Franklin Lakes, NJ). Following blood collection, mice were euthanized by cervical dislocation. 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.

2.3.4 Immunohistochemistry

Brains were removed and fixed in 5% acrolein in sodium phosphate buffer (0.1M, pH 7.4) at 20°C for 24 hours, followed by cryoprotection in 30% sucrose in phosphate-buffered saline (PBS: 0.05M, ph7.4) at 4°C until sectioning (at least 24 hours). Brains were sectioned (30µm) in the coronal plane with a cryostat and stored in a cryoprotectant solution (ethylene glycol/sucrose in sodium phosphate buffer) at -20°C until immunostained.

2.3.4.1 AVP Immunohistochemistry

Free-floating sections were rinsed five 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 placed in 0.1 M glycine in TBS for 30 min, rinsed again, and placed into block solution (10% normal goat serum (NGS), 0.4% Triton-X and 1% H2O2 in TBS) for 30 min. Sections were then incubated overnight(~18 hours) in anti-AVP (Bachem; 1:32000 dilution in TBS with 2% NGS and 0.4% Triton-X). The next day, sections were rinsed five times in TBS containing 1% NGS and 0.02% Triton-X and incubated in biotinylated secondary antiserum [goat anti-rabbit for AVP immunoreactivity (Vector Laboratories, Burlingame, CA)] diluted 1:250 in TBS with 2% NGS and 0.4% 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 four TBS 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.

2.3.4.2 OXT Immunohistochemistry

Sections were subjected to the same procedure outlined above, with the exception of the sodium citrate step, and the secondary antibody and ABC steps were increased to 90 minutes. Anti-OXT primary antibody (Peninsula Labs, 1:120,000) and goat anti-guinea pig secondary antibody (Vector Laboratories, 1:250) were used.

2.3.4.3 Iba-1 Immunohistochemistry

Sections were treated as outlined above with the following changes. Sections were washed nine times in TBS before a 60 min sodium citrate step. A concentrated blocking solution was used (TBS with 20% normal goat serum, 0.3% Triton-X, and 1% hydrogen peroxide), and sections were incubated overnight in rabbit anti-Iba-1primary antibody (Fisher, 1:20,000) diluted in TBS with 2% NGS and 0.3% Triton-X. Slices were then rinsed in dilute blocking solution (TBS with 1% NGS and 0.02% Triton-X) three times before secondary antibody.

2.3.5 Image Analysis

Matched sections for each mouse 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 or positively labeled cell bodies in the photomicrographs were gray-level threshold analyzed in Image J 1.43u (National Institutes of Health, Bethesda, MD) in accordance to the methods previously described in Rood et al., 2012. 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. Brain regions were selected from each of the three neuropeptide source and projection pathways: the PVN/SON pathway, the BNST-medial amygdala (MA) pathway, and SCN pathway (as described in Rood & De Vries, 2011). The PVN/SON pathway includes the PVN. The BNST-MA pathway includes the lateral habenula (LHb), ventral lateral septum (LS), and mediodorsal nucleus of the thalamus (MD). The SCN pathway includes the SCN, subparaventricular zone (SPZ), paraventricular nucleus of the thalamus (PVT), and the dorsomedial nucleus of the hypothalamus

(DMH). Cell counts were only available for OXT and Iba-1 staining, as the dense packing of cells and abundance of AVP-ir fibers made distinguishing individual cells impossible. The SON was not included in AVP-ir analysis because the staining was too dark to discern cell bodies or fiber tracts.

2.3.6 Statistical Analysis

Data were analyzed and visualized using IBM SPSS Version 21 (IBM). All data were analyzed by a two-way ANOVA with sex and treatment as factors, followed by Bonferroni post-hoc analyses. Differences in the post-hoc comparisons were noted as significant *$p<0.05$.

2.4 Results

2.4.1 Animals

All animals used in this study were in good health with no impairments. A total of 55 adult mice were used in the adult IHC experiment (9 male GF, 8 female GF, 10 male CC, 7 female CC, 13 male RE, and 8 female RE). In the weanling-aged IHC experiment, 29 mice were used (6 male GF, 9 female GF, 7 male CC, and 7 female CC). Finally, in the weanling-aged behavioral experiment, 63 mice were used (16 male GF, 17 female GF, 19 male CC and 11 female CC), except for the social interaction test, where 8 male CC and 4 female CC were excluded due to being paired with strangers, not littermates.

2.4.2 Adult Immunoreactivity

2.4.2.1 AVP Immunoreactivity

2.4.2.1.1 Suprachiasmatic Nucleus and Projection Sites

In the subparaventricular zone (SPZ), an effect of microbiota on AVP-ir emerged that resulted in a sex by treatment interaction (Figure 2.1A, $F(2, 53)= 5.880$, $p= 0.005$). In this region, male CC mice had higher AVP-ir expression than the CC females ($F(1, 53)= 8.961$, $p=0.004$). This sex difference was abolished in the GF mice but rescued in the RE mice. The overall levels of AVP-ir in RE mice were decreased compared to the CC mice (main effect of treatment, $F(2, 53)= 4.008$, $p= 0.025$; Bonferroni post-hoc analysis, $p= 0.052$).

The PVT showed a different AVP-ir expression pattern than the SPZ. RE mice had higher levels of AVP-ir than CC mice (Figure 2.1B; main effect of treatment, $F(5, 53)= 8.578$, $p=0.001$; Bonferroni post-hoc analysis, $p<0.001$) and a trend towards higher levels than GF mice ($p=0.059$). Males had consistently higher AVP-ir than females ($F(5, 53)= 9.038$, $p=0.004$).

There was no effect of germ-free status or recolonization on AVP-ir in the suprachiasmatic nucleus (SCN; Figure 2.1C, $p>0.05$). A projection site of the SCN, the DMH, also showed no differences between sex and treatment groups ($p>0.05$, data not shown).

2.4.2.1.2 Bed Nucleus of the Stria Terminalis-Medial Amygdala Pathway Projection Sites

In the lateral habenula (LHb), RE mice had greater AVP-ir than GF or CC mice (Figure 2.1D; $F(2, 54)= 8.532$, $p<0.001$, post-hoc analysis $p= 0.001$ and $p= 0.004$,

respectively). GF and CC males had higher levels of immunoreactivity than females ($F(1, 54)= 30.563$, $p<0.001$), and this sex difference was abolished in the RE mice. In the mediodorsal nucleus of the thalamus (MD), RE mice showed an increase in AVP-ir compared to CC mice (Figure 2.1E; $F(2, 52)= 5.278$, $p=0.009$; Bonferroni post-hoc analysis, $p=0.009$). Again, we replicated the sex difference seen in this region, in which males have a significant increase in immunoreactivity compared to the females ($F(1, 52)= 29.759$, $p<0.001$). In the lateral septum (LS), a projection site of the BNST, we replicated the well-established sex difference in AVP-ir ($F(1, 54)= 91.245$, $p<0.001$, data not shown), in which males had almost twice the immunoreactivity levels as the females in each treatment group (Gatewood et al., 2006; Rood et al., 2013).

2.4.2.1.3 Paraventricular Nucleus of the Hypothalamus

There was a trend towards a sex by treatment interaction on AVP-ir in the PVN (Figure 2.1F; $F(2, 54)= 3.012$, $p= 0.058$), where male RE mice had higher levels of AVP-ir than female RE mice ($p= 0.003$).

2.4.2.2 OXT Immunoreactivity

2.4.2.2.1 Paraventricular Nucleus of the Hypothalamus and Projection Areas

Germ-free mice had an increase in OXT-ir positive cells in the PVN compared to CC mice (Figure 2.2A; $F(2, 54)= 4.165$, $p=0.021$; Bonferroni post-hoc analysis, $p=0.013$). Recolonization with CC microbiota only partially returned the number of immunoreactive cells to CC levels. There was no difference in OXT-ir between groups in the PVN pixel number analysis despite an increase in OXT-ir cells in GF mice ($p>0.05$; data not shown).

Recolonization had differing effects on OXT-ir in the AH and PVT. In the AH, there was a trend towards RE mice having higher levels of OXT-ir than GF or CC mice (Figure 2.2B; $F(2, 54)=2.431$, $p=0.098$). In the PVT, a sex difference emerged in the RE mice, in which the females had higher immunoreactivity than the males (t-test, $p=0.003$). This sex difference was large enough to result in a trend towards an interaction between sex and treatment in the overall ANOVA (Figure 2.2C; $F(2, 54)= 2.926$, $p=0.063$).

Converse to the previous regions, there was a decrease in OXT-ir positive cells in the BNST in GF and RE mice (Figure 2.2D; $F(2, 54)= 4.178$, $p=0.021$; Bonferroni post-hoc analysis, $p=0.07$ and $p=0.087$, respectively). Despite the increase in OXT-ir positive cells, there was no difference in OXT-ir pixel number. There was a sex difference in the CC and a trend towards significance in GF groups, where females show more immunoreactivity than males ($F(1, 54)= 5.637$, $p=0.022$; t-test, $p=0.027$ and 0.096, respectively, data not shown).

There was no difference between treatment or sex in the SPZ ($p>0.05$).

2.4.2.2.2 Supraoptic Nucleus

Germ-free females had more OXT-ir positive neurons than the males, who had similar levels to the other groups (Figure 2.2E; t-test, $p=0.031$). Females had higher levels of OXT-ir than males, but this did not quite reach significance ($F(1, 39)= 3.167$, $p=0.084$).

2.4.2.3 Iba-1 Immunoreactivity

In the BNST, there was a sex by treatment interaction in Iba-1 immunoreactivity (Figure 2.3A; $F(2, 52)= 5.883$, $p=0.005$), driven by greater immunoreactivity in male CC mice, a reversal of the sex difference in the GF mice, and a decrease in

immunoreactivity in the GF and RE mice compared to CC mice (Bonferroni post hoc analysis, p=0.05 and p=0.003, respectively). Iba-1 positive cell counts in the BNST showed a similar pattern to Iba-1 immunoreactivity (Figure 2.3E; sex by treatment interaction, $F(2, 52)= 6.511$, $p= 0.003$), including the reversal of the sex difference in the GF mice, but there were no significant differences between treatment groups (Bonferroni post-hoc analysis, $p>0.05$). This pattern of sex differences and decrease from CC mice persisted in Iba-1 positive cell counts, resulting in a sex by treatment interaction ($F(2, 37)= 5.233$, $p=0.011$). CC mice had higher cell counts than RE mice ($p=0.034$).

There was a sex by treatment interaction in Iba-1 immunoreactivity in the LS (Figure 2.3B; $F(2, 37)= 4.059$, $p= 0.027$), driven partially by the appearance of a sex difference in GF mice. GF mice and RE mice had decreased immunoreactivity compared to CC mice (Bonferroni post-hoc analysis, $p=0.027$ and $p=0.003$, respectively). There was a trend towards a sex by treatment interaction in Iba-1 positive cell counts (Figure 2.3F; $F(2, 37)= 2.913$, $p=0.069$), driven by a sex difference in the GF mice, with females showing higher numbers of microglia than males (main effect of sex, $F(1, 37)= 4.972$, $p=0.033$).

In the striatum, there was a trend towards a sex by treatment interaction (Figure 2.3C; $F(2, 37)= 3.051$, $p=0.061$) in Iba-1 immunoreactivity. CC mice had higher levels of immunoreactivity than GF or RE mice ($F(2, 37)= 9.5$, $p=0.001$, Bonferroni post-hoc analysis, $p=0.044$ and $p<0.001$, respectively). There was an increase in immunoreactivity in the males of CC mice and RE mice, but not in the GF animals, contributing to a trend towards a main effect of sex ($F(1, 37)= 3.509$, $p=0.07$). This

pattern of sex differences and decrease from CC mice persisted in Iba-1 positive cell counts, resulting in a sex by treatment interaction (Figure 2.3G; $F(2, 37)= 5.233$, $p=0.011$). CC mice had higher cell counts than RE mice (t-test, $p=0.034$).

There was a sex by treatment interaction in Iba-1 immunoreactivity in the PVT (Figure 2.3D; $F(2, 51)= 6.080$, $p=0.005$), driven by greater immunoreactivity in the male CC mice ($p= 0.001$). CC mice had higher numbers of Iba-1 positive microglia compared to both GF and RE mice (Figure 2.3H; $F(2, 51)= 4.123$, $p= 0.023$, Bonferroni post-hoc analysis, $p= 0.019$ and $p= 0.039$, respectively), driven by a similar increase in male CC Iba-1 cells ($p= 0.006$), leading to an almost significant main effect of sex ($F(1, 51)= 3.945$, $p=0.053$). GF and RE mice did not have a sex difference in Iba-1 expression, indicating that this sex difference is established by microbiota exposure before weaning.

There were no treatment differences in Iba-1 immunoreactivity nor Iba-1 positive cell count in the PVN ($p>0.05$).

2.4.3 Weanling-Aged Immunoreactivity

Due to the above results, showing that recolonization does not rescue GF mice to expression levels of CC mice, we were interested in whether weanling-aged GF mice show similar deficits in AVP, OXT and Iba-1 expression. We first examined AVP, OXT, and Iba-1 expression in weanling-aged mice to establish whether changes to these systems are present at weaning. In a cohort, we established the behavioral and morphological profile of weanling-aged mice, to determine if any changes in these neural circuits correlate with behavioral changes.

2.4.3.1 AVP Immunoreactivity

2.4.3.1.1 Suprachiasmatic Nucleus and Projection Sites

In the SCN, GF mice showed less immunoreactivity than the CC mice (Figure 2.4A; $F(3, 28)= 15.877$, $p=0.001$). In a projection site from the SCN, the DMH, there was a similar decrease in AVP-ir in the GF mice (Figure 2.4B; $F(3, 28)=5.954$, $p=0.022$). There was no effect of germ-free status on AVP-ir in the SPZ ($p>0.05$; data not shown).

A different pattern was seen in the anterior portion of the PVT. GF mice had higher AVP-ir than CC mice (Figure 2.4C; $F(3, 28)= 6.179$, $p=0.02$), driven by a substantial increase in AVP-ir in the females (sex by treatment interaction, $F(3, 28)= 5.733$, $p= 0.024$).

2.4.3.1.2 Paraventricular Nucleus of the Hypothalamus

In the PVN, the GF mice had higher levels of AVP-ir than the CC mice, but this difference did not reach significance (Figure 2.4D; $F(3, 28)= 2.992$, $p= 0.096$).

2.4.3.1.3 Bed Nucleus of the Stria Terminalis and Projection Sites

At weaning, there was no visible staining in the lateral septum, lateral habenula or mediodorsal nucleus of the thalamus with the antibody for AVP used in this study, thus we were unable to quantify AVP-ir in these regions.

2.4.3.2 OXT Immunoreactivity

2.4.3.2.1 Paraventricular Nucleus of the Hypothalamus and Projection Sites

Germ-free mice had increased OXT-ir compared to CC mice in the PVN ($F(1, 28)= 21.946$, $p<0.001$, data not shown). This may be partially attributed to an increase in OXT-ir positive cells in GF mice (Figure 2.5A, $F(1, 28)= 21.54$, $p<0.001$).

There was no effect of the lack of microbiota on OXT fiber projections from the PVN in the anterior hypothalamus (AH; Figure 2.5B), PVT (Figure 2.5C), DMH and SPZ (data not shown; $p>0.05$). Females had increased OXT-ir in the BNST compared to males ($F(1, 28)= 7.171$, $p=0.013$, data not shown). However, there was an interaction of sex and treatment in OXT-ir positive cells in the BNST (Figure 2.5D; $F(1, 27)= 4.608$, $p= 0.042$), where GF males had a trend towards increased OXT-ir cells than GF females ($p=0.077$).

2.4.3.2.2 Supraoptic Nucleus

Germ-free mice had an increased number of OXT-ir positive cells in the SON (Figure 2.5E; $F(1, 28)= 5.611$, $p=0.026$). This did not extend to an increase in OXT-ir in the SON area analyzed, however ($p>0.05$).

2.4.3.3 Iba-1 Immunoreactivity

GF mice showed less Iba-1 immunoreactivity (Figure 2.6A; $F(1, 26)= 4.584$, $p=0.043$) and less microglia cell counts ($F(1, 26)= 9.656$, $p=0.005$, data not shown) than CC mice in the BNST. There was a sex by treatment interaction in the striatum (Figure 2.6C; $F(1, 19)= 6.937$, $p=0.018$), in which the sex difference in Iba-1 immunoreactivity in the CC mice was abolished in GF mice. This interaction was driven by the greater immunoreactivity in CC males. Microglia counts were decreased in the striatum in GF

mice compared to CC mice ($F(1, 19)= 21.127$, $p<0.001$, data not shown), and there was a trend towards a main effect of sex ($F(1, 19)= 3.336$, $p=0.087$). In the PVN, there were no effects of sex or treatment on Iba-1 immunoreactivity, but there was a trend towards a sex by treatment interaction in microglia count ($F(1, 27)= 3.219$, $p=0.085$, data not shown). There were no differences in sex or treatment in Iba-1 positive cells or immunoreactivity in the LS (Figure 2.6B) or PVT (Figure 2.6D; $p>0.05$).

2.4.4 Weanling-Aged Behavior and Body Measures

2.4.4.1 Social Behavior

GF mice spent less time interacting with a familiar mouse in the social interaction test than CC mice, (Fig. 2.7A, $F(3, 59)= 19.149$, $p<0.001$). Male CC mice showed similar levels of social interaction as both male and female GF mice, indicating that a lack of microbiota abolished the sex difference seen in the CC mice, whereas female CC mice spent more time socially with the target mouse, resulting in a main effect of sex, ($F(3, 59)= 12.343$, $p<0.001$), and an interaction between sex and treatment ($F(1, 59)= 13.457$, $p<0.001$). This same pattern was seen in allogrooming behavior, where GF mice also spent less time allogrooming than CC female mice, but more than the CC males, resulting in a sex by treatment interaction (Fig. 2.7B, $F(3, 17)= 9.052$, $p= 0.009$).

There was an interaction between sex and treatment in time spent walking in the arena (Fig. 2.7C, $F(3, 59)= 6.751$, $p= 0.012$). This was driven by a reversal in the direction of sex differences from CC males walking more to GF females walking more. When not walking or interacting with the other mouse, GF mice spent their time rearing, (Fig. 2.7D; $F(3, 56)= 7.758$, $p=0.007$), and showed a trend towards spending more time grooming than CC mice, ($F(3, 57)= 3.602$, $p= 0.063$; data not shown). There

was no difference between GF and CC mice in time spent immobile or time spent digging in the bedding ($p>0.05$; data not shown).

2.4.4.2 Marble Burying Test

Conventionally colonized males spent more time digging than CC females in the marble burying test (Fig. 2.8A; $F(3, 74)= 4.788$, $p=0.032$), but this sex difference is abolished in the GF mice. Both GF and CC mice walked in the arena similar amounts of time ($p>0.05$; data not shown), but the main difference lied in their behavior when not walking and digging. GF mice spent more time immobile (Fig. 2.8B; $F(3, 74)= 15.268$, $p<0.001$), in which they were not actively investigating the arena, versus the CC mice, who spent more time rearing against the walls of the arena (Fig. 2.8C; $F(3, 74)= 11.647$, $p=0.001$).

2.4.4.3 Elevated Plus Maze

Weanling aged GF mice showed decreased anxiety behavior in the elevated plus maze, as measured by time spent in the open arms (Figure 2.9A; $F(3, 74)=8.039$, $p=0.006$). This difference was due to GF mice spending more time in the outer half of the open arms of the apparatus than CC mice (Figure 2.9B; $F(3, 62)=10.898$, $p=0.002$), but not due to an increase in distance traveled in the GF mice (Figure 2.9C; $p>0.05$). There was no difference in the time spent immobile in the apparatus between the GF and CC mice ($p>0.05$; data not shown).

2.4.4.4 Body Measures

Overall, weanling GF mice weighed less than the CC mice (Fig. 2.10A, $F(3, 44)= 40.43$, $p<0.001$), driven by smaller gonadal adipose deposits (Fig. 2.10B, $F(4, 62)= 31.431$, $p<0.001$). Males of both treatments had larger gonadal fat pads than the

females, despite being prepubertal (Fig. 2.10B, $F(4, 62)= 10.237$, $p=0.002$). As previously reported, GF mice had ceca that are twice as heavy as the CC mice to aid in digestion (Fig. 2.10C, $F(4, 62)= 126.705$, $p<0.001$). Furthermore, GF mice did not show morphological markers of inflammation, demonstrated by a trend towards smaller spleens and shorter colons than the CC mice (Fig. 2.10D, $F(4, 62)= 2.97$, $p= 0.09$; data not shown, $F(3, 44)= 14.699$, $p<0.001$, respectively). GF mice showed a sex difference in colon weight, with females having heavier colons than males (data not shown, $F(4, 62)= 4.647$, $p=0.035$).

2.5 Discussion

In this study, we demonstrated that the lack of microbiota alters AVP, OXT and microglia at weaning and in adulthood, but not necessarily in the same manner. Specifically, AVP-ir was decreased in weanling-aged GF mice, but there were no differences between GF and CC animals in adulthood, whereas OXT-ir was higher in both weanling-aged and adult mice. Furthermore, Iba-1 immunoreactivity generally decreased in GF mice, but this was sex- and region-specific. Recolonization was not sufficient to restore CC levels of immunoreactivity for all three neural markers, although it did increase AVP-ir in some regions and rescue some sex differences observed in the CC mice. The changes in these neural systems were accompanied by reduced anxiety-like and social behavior in weanling-aged mice. These results suggest that microbiota is necessary for proper development of microglia and AVP systems, and changes to the microbiota during development may lead to behavior alterations downstream.

Very few experiments have investigated the effects of microbiota on AVP, OXT, and microglia, and we wanted to investigate how and where germ-free status affects

these systems. While we cannot use these results to make causative claims on the interaction between the microbiota, neuropeptides, and behavior, we can now use these results to perform hypothesis-driven experiments to elucidate the mechanism as to how microbiota contributes to the development of these systems. We also did not replicate the adult GF behavioral profile to prevent behavioral testing-induced changes in their neurochemistry (Gagliano et al., 2008). However, the behavioral phenotype of GF mice is well-established (reviewed in Luczynski et al., 2016) and the behavioral profile we observed in the weanling-aged mice is similar to that published in adult GF mice.

We found that AVP immunoreactivity decreased in the SCN and its projections in juvenile GF mice. In addition to its role in the control of circadian rhythms, the SCN and its projection sites modulate the HPA axis and depressive-like behavior (reviewed in Kalsbeek et al., 2010). AVP derived from the parvocellular neurons of the SCN have an inhibitory effect on the HPA axis, and AVP release in the DMH from the SCN is responsible for lowering circulating levels of corticosterone during the first half of the light period (Kalsbeek et al., 1996; Kalsbeek et al., 1992). Thus, the decreased AVP-ir we found may indicate more released AVP, which may reduce the level of corticosterone in circulation. This would make sense, as GF mice have reduced anxiety-like behavior than CC mice, but elevated HPA axis activation (Sudo et al., 2004). Conversely, AVP-ir was increased in the PVT. The PVT is involved in a number of behaviors, including food intake, food reward, emotional regulation and circadian rhythms, due to the variation in inputs and projections from this region (Kirouac, 2015). AVP has excitatory effects on PVT neurons (Zhang et al., 2005), so the effects of the increased AVP in the PVT is dependent on the recipients of the excitatory

signaling. It is possible that its projections to the nucleus accumbens would increase the reward from food intake or would increase anxiety-like behavior through its activation of the extended amygdala (Heydendael et al., 2011; Stratford and Wirtshafter, 2013). However, the posterior PVT is heavily innervated by orexin neurons that increase anxiety when activated (Heydendael et al., 2011), suggesting that the anxiogenic role of the PVT is more posterior than the anterior region we analyzed. Finally, juvenile GF mice showed a trend towards an increase in AVP-ir in the PVN, which suggests that there may be alterations to the production of AVP or that the slight increase may contribute to the altered social and anxiety-like behavior in these mice.

In the adult mice, we found that AVP-ir was increased in the RE mice in the PVT, LHb, and MD compared to CC mice. In general, there was a slight increase in the GF mice, but it was not significantly different from CC or RE mice. We expect that the increase AVP-ir in the RE mice is due to the combination of early-life stress of oral gavage, the immune stress of encountering microbiota for the first time, and the normal stress of weaning interacting to affect AVP-ir permanently. Early life stress increases AVP-ir in males in the PVN and AVP receptor 1a (V1aR) binding in the amygdala and hypothalamus (Veenema et al., 2007). These studies suggest that the maternal separation that occurs during weaning may affect AVP system function in adulthood. Furthermore, colonization of GF mice corrects the deficits in mucosal immune system function (Umesaki et al., 1995), but little examination of how the immune stress of colonization impacts brain development has been done. It would be interesting to examine the behavior of the recolonized mice to see if it correlates with the increased AVP-ir. In the SPZ, we saw a decrease in AVP-ir in the RE mice. The

SPZ receives AVP projections from the SCN, and the SPZ is involved in controlling body temperature rhythms, sleep rhythms and locomotor activity, with downstream functions in energy homeostasis and HPA axis regulation (Vujovic et al., 2016). The reduction in AVP-ir in the SPZ may be due to increased AVP release from the SPZ or decreased production. Reduced production may explain the anxiolytic nature of GF mice, due to decreased HPA axis activation. Alternatively, increased release of AVP may be acting on other neural systems to result in the behavioral changes.

In the PVN, we only saw trends towards an effect of treatment on AVP-ir in both adults and weanling-aged mice. It is possible that GF mice would show differences in immunoreactivity in projection sites from the PVN, similar to our results in the SCN and its projection sites. More investigation into the PVN and its projections is needed to understand how the lack of a microbiota impacts this AVP pathway.

We found increased OXT-ir in the PVN and SON of juvenile mice. OXT production and release are activated by stressful stimuli and has an anxiolytic effect on the brain. OXT administration either by ICV or into specific brain regions like the PVN results in decreased anxiety-like behavior in both rats and mice (Ring et al., 2006; Blume et al., 2008; Jurek et al., 2012). An increase in OXT production or release may partially explain the anxiolytic behavior patterns of GF mice. A similar pattern is seen in the adults, where GF mice have more OXT positive cells in the PVN. Of interest, there was a trend towards a decrease in OXT positive cells in the BNST. OXT actions in the BNST mediates social recognition, social vigilance, and acquisition of cued fear (Dumais and Veenema, 2016; Moaddab and Dabrowska, 2017; Duque-Wilckens et al., 2018), suggesting that the decrease in this region may contribute to the deceased social

behaviors seen in GF mice (Desbonnet et al., 2014). Alternatively, there may be less release of OXT, resulting in more OXT-ir in the PVN and SON, suggesting that the behavioral changes we saw may be due to OXT's effects on the PVN and SON specifically.

Our microglial results were divergent from those in previous experiments. We found that mice lacking a microbiota had less microglial immunoreactivity and number, measured as Iba-1 positive cells, whereas others report that GF mice have an immature microglial phenotype, characterized by an increase in microglial number and increased ramification of protrusions (Erny et al., 2015; Castillo-Ruiz et al., 2018). Furthermore, Thion and colleagues reported that female microglia are more perturbed by the lack of microbiota in adulthood, whereas males were more affected prenatally, which we did not replicate (Thion et al., 2018). In fact, we found that GF females showed overall similar levels of microglia expression to CC females and were affected by recolonization more than males. Male GF mice showed a decrease in microglia compared to CC mice, and in general were not affected by recolonization. A few factors may account for these differences. One, our experiments used Swiss-Webster mice as opposed to the C57Bl/6 mice used in the previous studies. Another factor is that we did not measure cytokine expression or microglial activation level, so there may be other factors underlying these differences that are unknown. The fact that recolonization did not recapitulate the CC phenotype is interesting, because Erny and colleagues report that colonization with complex microbiota could restore deficient microglia (Erny et al., 2015). However, they recolonized the GF mice in their experiment in adulthood, suggesting the timing of recolonization may be crucial to rescue the abnormal microglia

in GF mice. Taken together, it is clear that more research must be done in order to tease apart the temporal factors that connect microglia and microbiota.

Our main behavioral finding is that weaning-aged GF mice show less anxiety-related behavior than CC mice. This change in basal anxiety-like behavior is well supported in the literature, where adult GF mice spent more time in the open arms of the EPM compared to SPF mice (Diaz Heijtz et al., 2011; Neufeld et al., 2011b, 2011a; Clarke et al., 2013). Interestingly, Lu and colleagues (2018) found that GF mice spent less time in the center of the open field area and no difference in time spent in the closed arms of the EPM for C57Bl/6 mice at 4 weeks of age (Lu et al., 2018). As our study used Swiss-Webster mice, as did the experiments done by Neufeld and Clarke, the differences in results suggest these differences may be due to the effects of mouse strain. It is worth noting that GF mice in our study showed decreased anxiety behavior at weaning, which suggests that these patterns of behavior are developing before puberty, and that puberty may be a critical period for microbiota's effects on anxiety-like behavior. Evidence for this is seen in the addendum by Neufeld and colleagues (2011), who demonstrated that colonization of GF mice in adulthood was not sufficient to rescue the anxiolytic phenotype of GF mice. Mice that were treated with a cocktail of antibiotics form weaning to adulthood to produce a model similar to germ-free mice showed an anxiolytic phenotype as well, further suggesting that puberty is a sensitive period for effects of microbiota (Desbonnet et al., 2015).

We found that our juvenile GF mice showed deficiencies in social behavior in a sex-dependent manner. Our findings are partially supported by experiments by Desbonnet et al. (2014), in which they found that male GF mice spent less time

investigating a novel mouse in the three-chamber sociability test than CC males, whereas we observed that only female GF mice were less social than female CC mice (Desbonnet et al., 2014). There are a number of factors that may account for this difference. Our study used weanling-aged mice in a different behavioral assay, suggesting that age and experimental paradigm are important factors in the expression of social behavior in microbiome manipulation studies. Other studies found increased sociability (Arentsen et al., 2015) or no difference (Lu et al., 2018) between GF and CC mice. These studies differed in the strain of stimulus mouse used, age of subjects, and use of both sexes. The contradiction in results from social behavior assays using germ-free mice further supports the importance of considering mouse strain, age, and experimental paradigm (such as using littermates versus novel mice) when interpreting behavioral results.

The reduction in social behavior in GF mice may be due to changes in odor cues and odor processing. We used either two GF or two CC mice in our social interaction paradigm to control for differences what odor signals the mouse receives and how they are processed. Interestingly, Singh and colleagues demonstrated that rats cannot discriminate between urine of two genetically dissimilar GF rats (Singh et al., 1990), nor between two GF mice (Schellinck et al., 1995), suggesting that microbiota may be necessary for individual odors to develop. However, mice can be trained to differentiate between two germ-free mice in the Y-maze task, indicating that not all individual odor is due to microbial factors, or that not all differentiation between animals is olfactory (Yamazaki et al., 1990). Furthermore, recent evidence suggests that GF mice have reduced olfactory epithelium cilia thickness and cellular turnover, reduced olfactory

transduction expression genes, and a stronger electro-olfactogram response to number of odorants, which may alter how a GF mouse may receive and process social odors (François et al., 2016). Thus, the discrepancy in social behavior between our and other studies may be due to differences in odor cues and processing.

We found that male CC mice spent more time digging in the marbled arena than female CC mice, but this sex difference was abolished in GF mice. Males have been shown to bury more marbles females in some studies (Mitra et al., 2018), but most do not report sex differences in number of marbles buried (Kokras and Dalla, 2014; Taylor et al., 2017; Tucker and McCabe, 2017). These studies used the number of marbles buried instead of time spent digging, meaning we may have captured more subtle sex differences than can be seen in the number of marbles buried alone to explain our findings. Alternatively, the age of our mice and experimental procedure may account for the differences. We used weanling-aged mice and only tested in the arena for 10 minutes, when most experiments tested adults for 30 minutes (Çalişkan et al., 2017). It is possible that males dig more in the first 10 minutes of testing than females, but the difference disappears over the full 30 minutes. Thus, something about the lack of microbiota in males may decrease the amount of digging seen in the first 10 minutes of the marble burying test.

Our results show that GF upbringing is associated with changes to AVP and OXT expression, as well as dysfunctional microglial response to microbiota colonization. These results show that microbiota is necessary for proper development of the AVP and OXT systems, and microbiota deficiency neonatally may alter the organization these systems that results in long term behavioral changes. More

evidence is required to determine when and through what pathways the lack of microbiota affects AVP and OXT development, and what implications this has on behavior. Understanding the mechanisms underlying this phenomenon may provide novel therapeutics for psychiatric and neurodevelopmental disorders with anxiety and social deficit components.

2.6 Figures

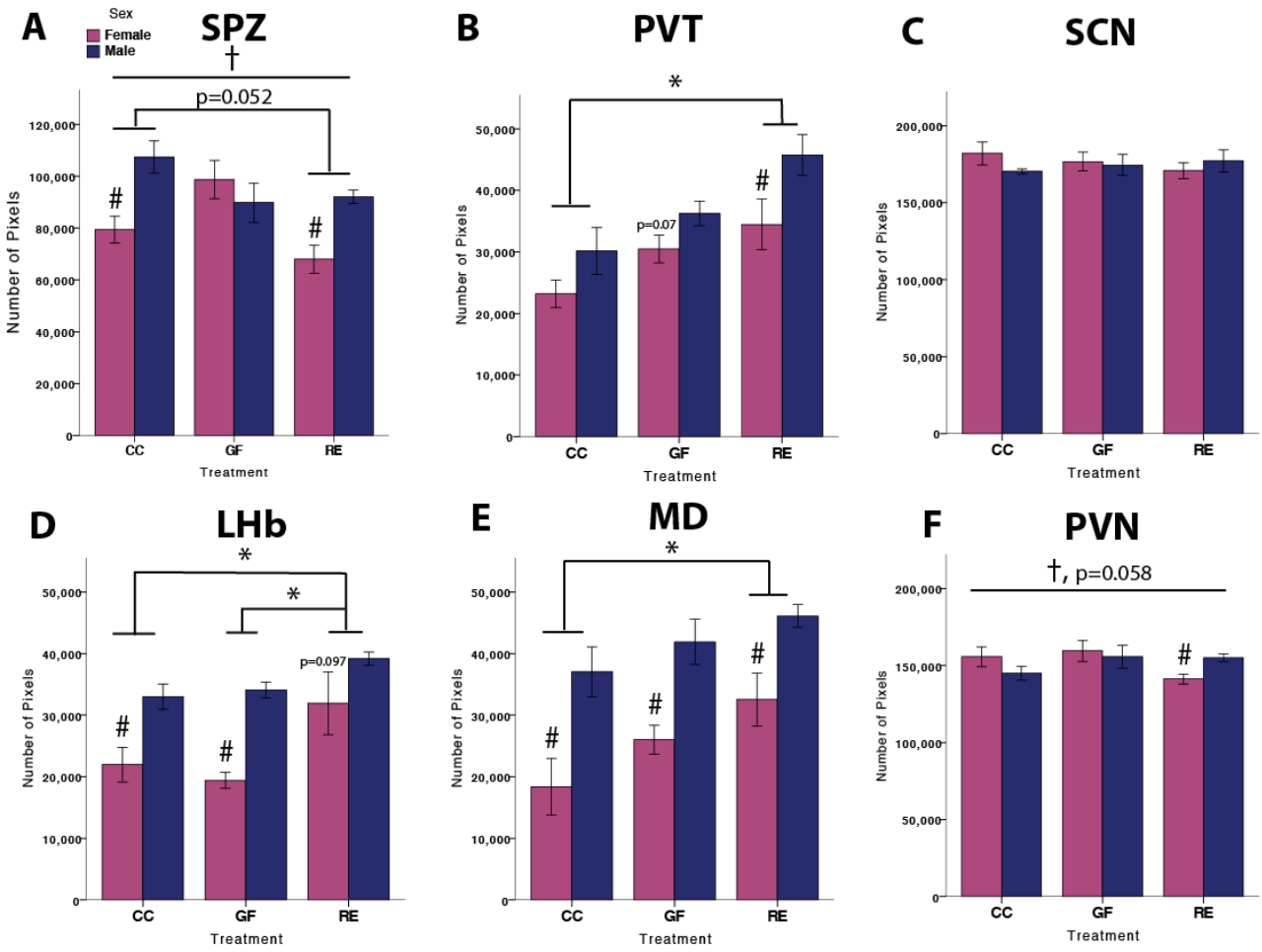

Figure 2.1. Recolonization with microbiota generally increases AVP immunoreactivity in adult mice.

Graphs depict AVP immunoreactivity (number of pixels above threshold) in the **(A)** SPZ, **(B)** PVT, **(C)** SCN, **(D)** LHb, **(E)** MD, and **(F)** PVN. * represents a significant main effect of treatment, with significant post-hoc tests indicated ($p<0.05$). # represents a significant sex difference when the ANOVA indicates a main effect of sex ($p<0.05$). † represents a significant treatment by sex interaction ($p<0.05$). Trends toward significance are represented with the p-value. Data presented as +/- SEM (n=7-13).

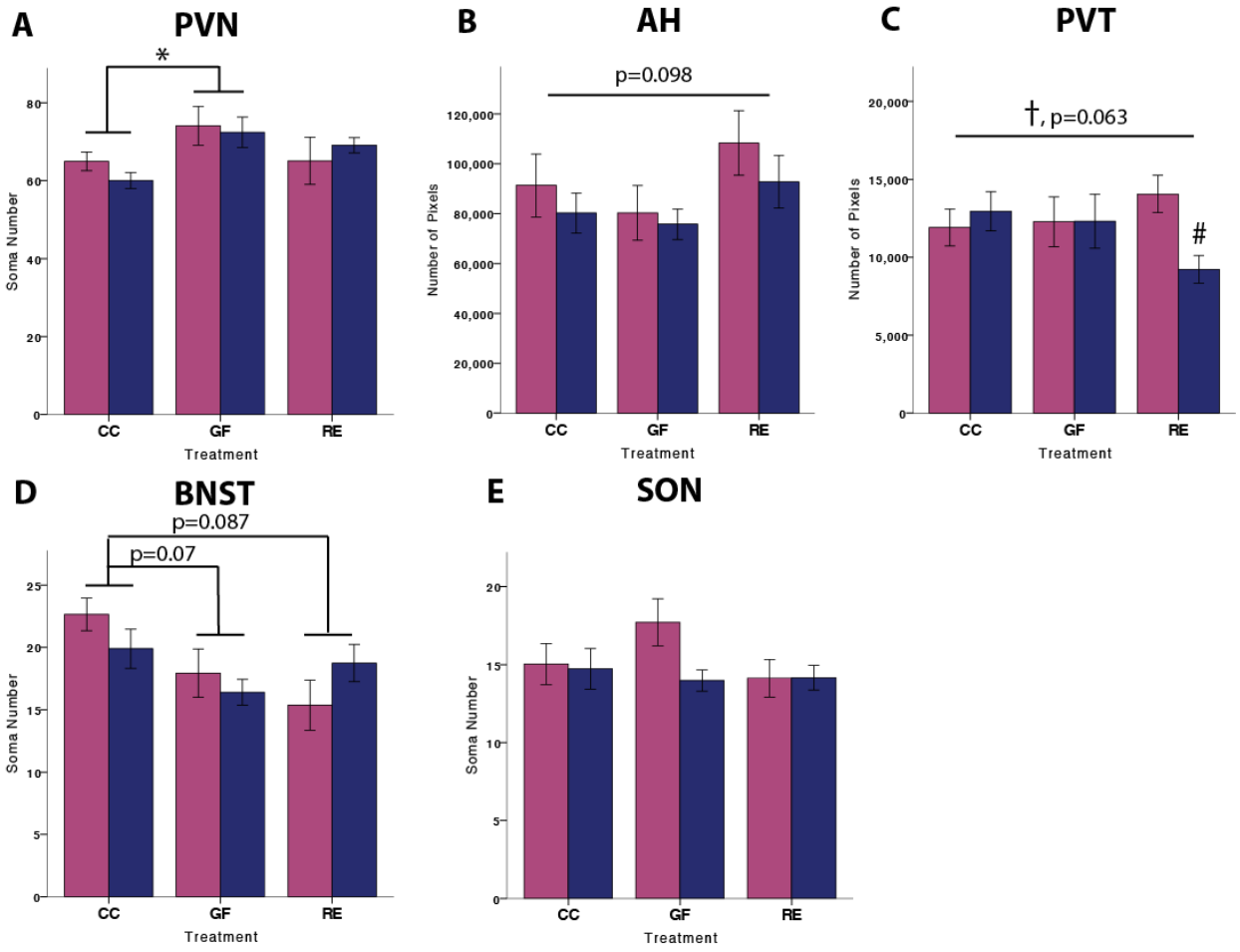

Figure 2.2. Microbiota alter adult OXT-ir in sex- and region-specific ways.

Graphs depict OXT immunoreactivity in the **(A)** PVN soma number, **(B)** AH immunoreactivity, **(C)** PVT immunoreactivity, **(D)** BNST soma number, and **(E)** SON soma number. * represents a significant main effect of treatment, with significant post-hoc tests indicated ($p<0.05$). # represents a significant sex difference when the ANOVA indicates a main effect of sex ($p<0.05$). ┼ represents a significant treatment by sex interaction ($p<0.05$). Trends toward significance are represented with the p-value. Data presented as +/- SEM (n=7-13).

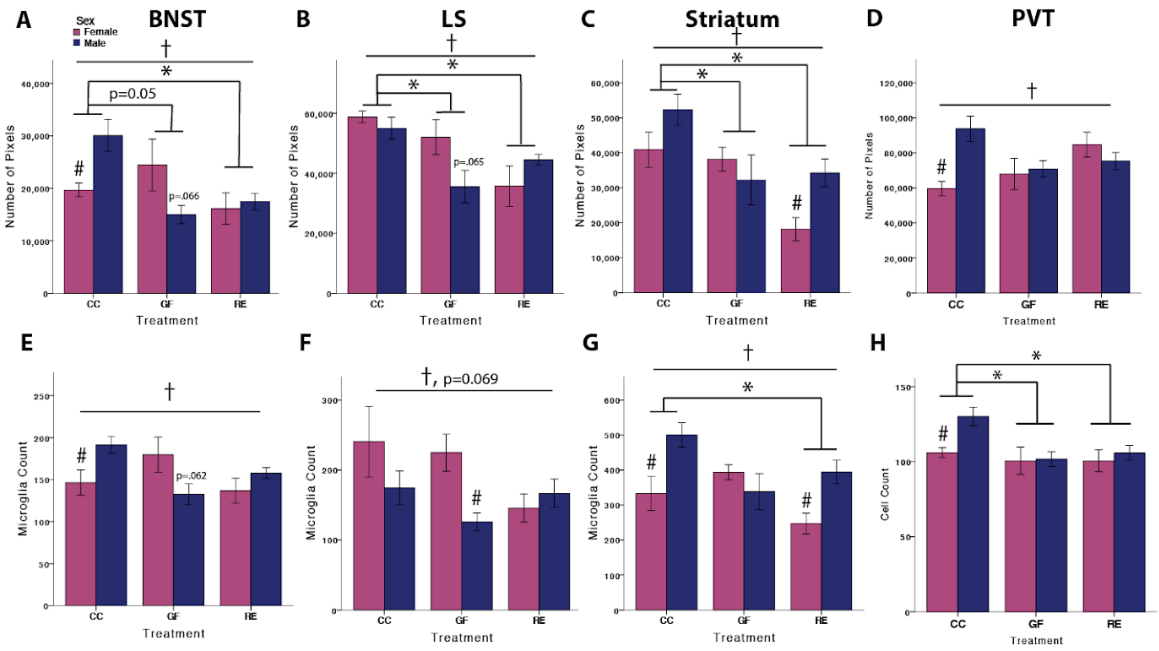

Figure 2.3. Recolonization does not rescue the reduced microglia expression in GF mice.

Graphs depict Iba-1 immunoreactivity **(A-D)** or Iba-1 positive cells (**E-H)** in the **(A, E)** BNST, **(B, F)** LS, **(C, G)** striatum, and **(D, H)** PVT. * represents a significant main effect of treatment, with significant post-hoc tests indicated ($p<0.05$). # represents a significant sex difference when the ANOVA indicates a main effect of sex ($p<0.05$). † represents a significant treatment by sex interaction ($p<0.05$). Trends toward significance are represented with the p-value. Data presented as +/- SEM (n=7-13).

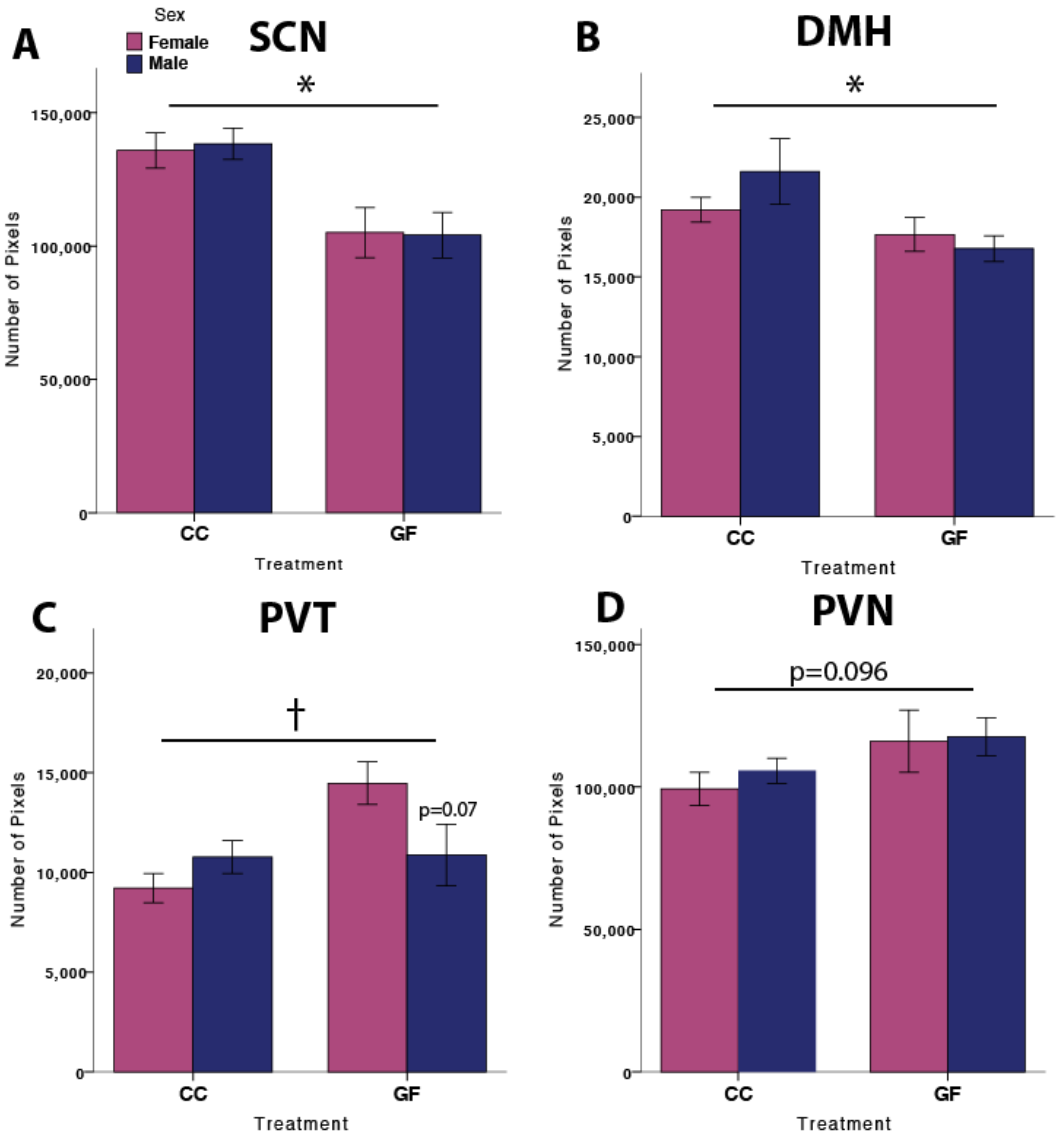

Figure 2.4. Lack of a microbiota alters AVP-ir at weaning in a region-specific manner.

Graphs depict AVP-ir in the **(A)** SCN, **(B)** DMH, **(C)** PVT, and **(D)** PVN. * represents a significant main effect of treatment ($p<0.05$). ┼ represents a significant treatment by sex interaction ($p<0.05$). Trends toward significance are represented with the p-value. Data are represented as means ± SEM (n=6-9).

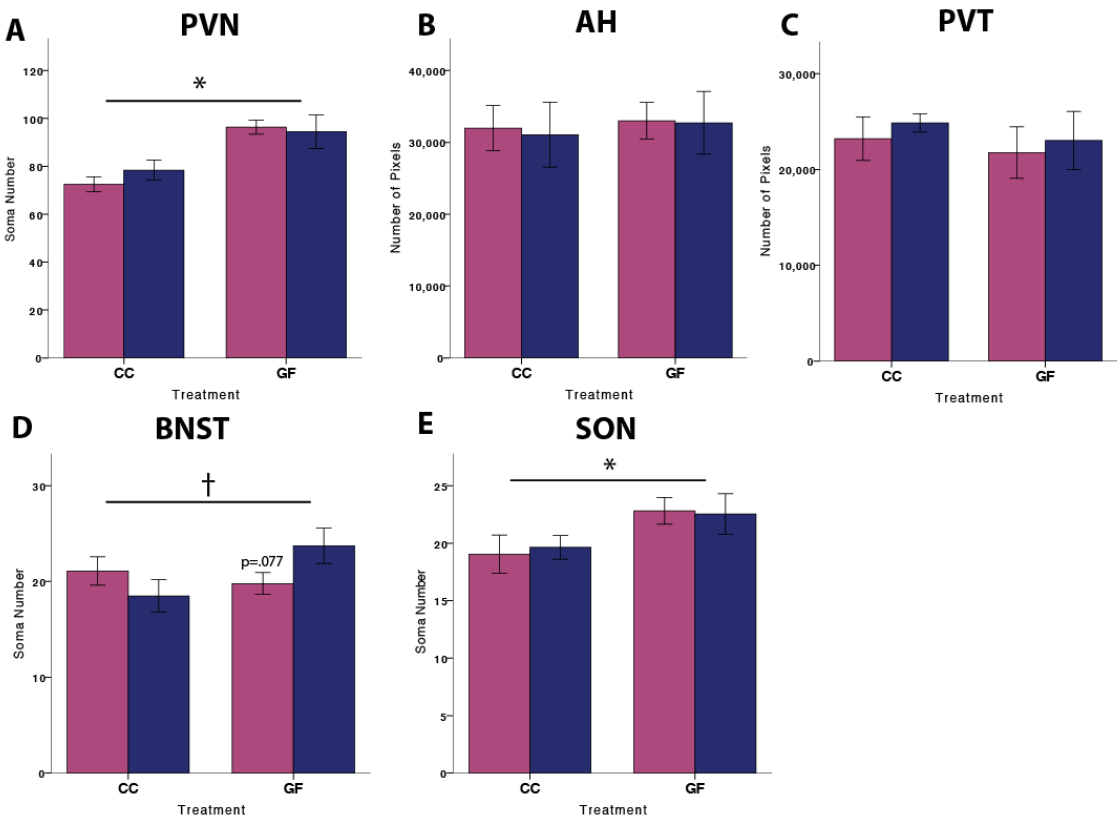

Figure 2.5. Germ-free conditions in weanling-aged mice increase OXT-ir in some brain regions in a similar pattern to adults.

Graphs depict OXT-ir **(B, C)** or OXT positive cells **(A, D, E)** in the **(A)** PVN, **(B)** AH, **(C)** PVT, **(D)** BNST, and **(E)** SON. * represents a significant main effect of treatment ($p<0.05$). ┼ represents a significant treatment by sex interaction ($p<0.05$). Trends toward significance are represented with the p-value. Data are represented as means ± SEM (n=6-9).

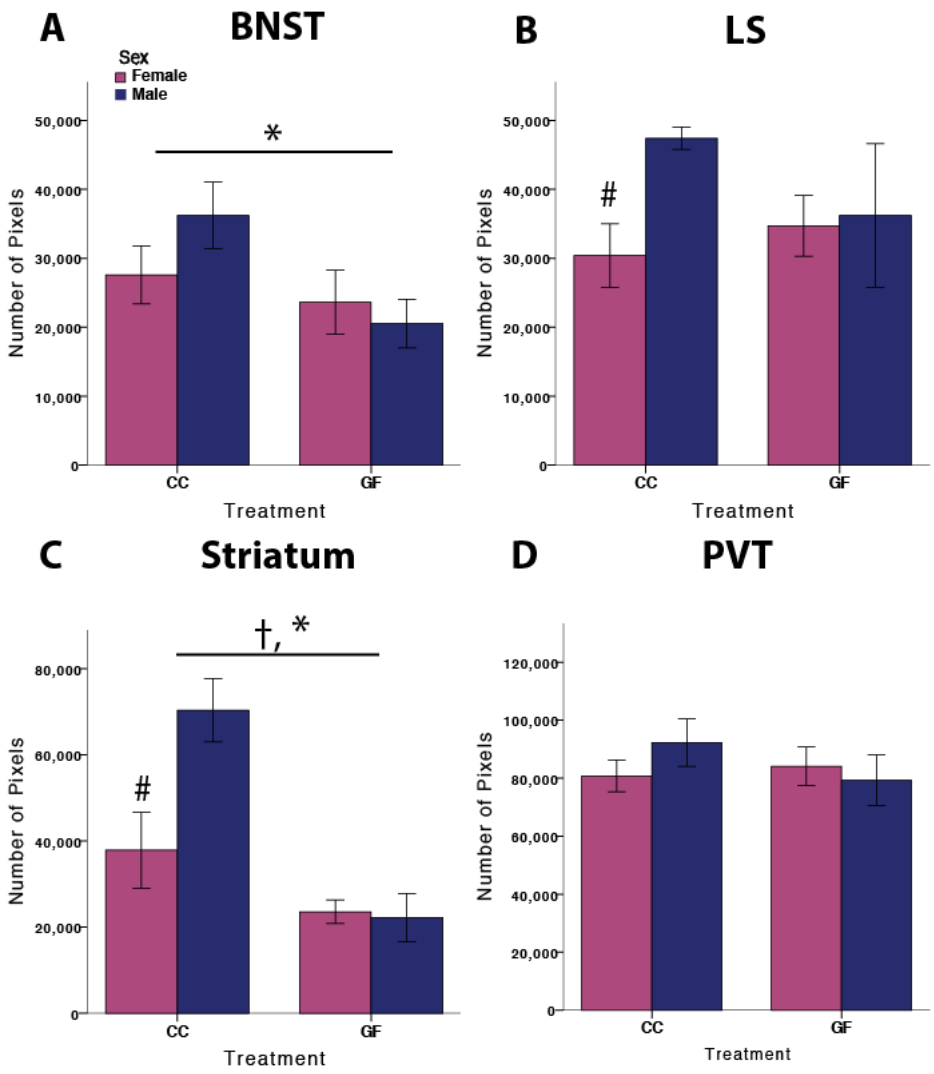

Figure 2.6. Germ-free conditions reduce microglia expression in a brain region-dependent manner.

Graphs depict Iba-1 immunoreactivity in the **(A)** BNST, **(B)** LS, **(C)** striatum, and **(D)** PVT. * represents a significant main effect of treatment ($p<0.05$). # represents a significant sex difference when the ANOVA indicates a main effect of sex ($p<0.05$). † represents a significant treatment by sex interaction ($p<0.05$). Data are represented as means ± SEM (n=6-9).

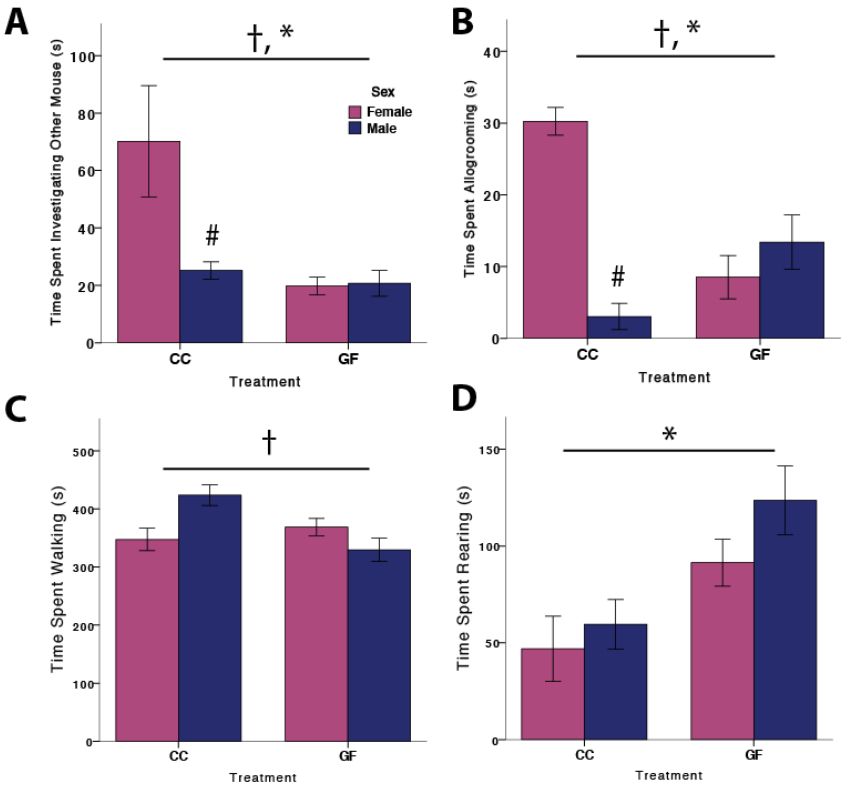

Figure 2.7. Female GF mice spend less time interacting with a littermate than female CC mice at weaning.

Graphs depict **(A)** Time spent actively investigating their littermate, **(B)** time spent grooming the other mouse, **(C)** time moving around the arena, and **(D)** time spent rearing against the side of the cage. * represents a significant main effect of treatment ($p<0.05$). # represents a significant sex difference when the ANOVA indicates a main effect of sex ($p<0.05$). ┼ represents a significant treatment by sex interaction ($p<0.05$). Data are represented as means ± SEM (n=10-22).

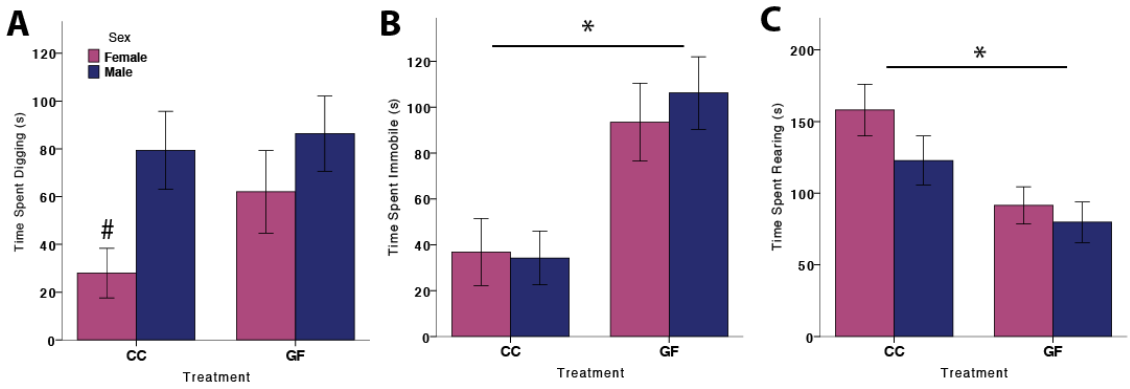

Figure 2.8. GF mice do not dig more in the marble burying test but spend more time immobile.

(A) time spent digging in the bedding in the arena, **(B)** time spent sitting immobile in the arena, and **(C)** time spent rearing against the cage walls. * represents a significant main effect of treatment ($p<0.05$). # represents a significant sex difference when the ANOVA indicates a main effect of sex ($p<0.05$). Data are represented as means ± SEM (n=10-22).

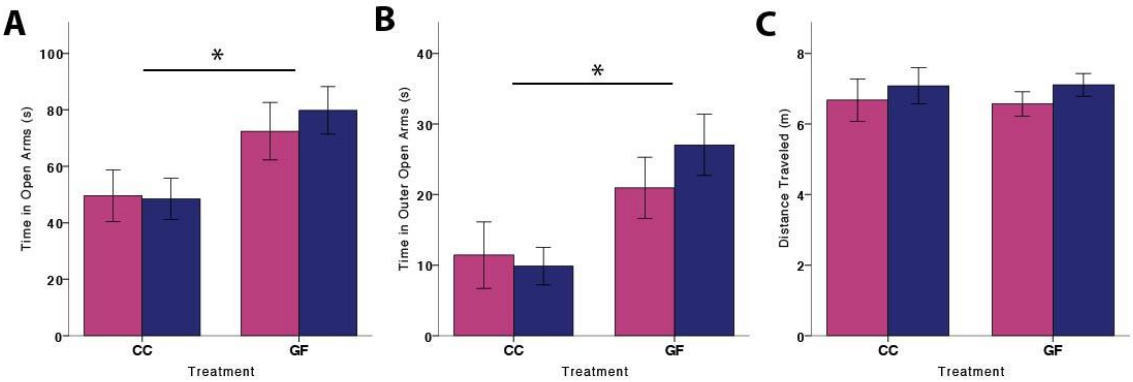

Figure 2.9. Weanling-aged GF mice show less anxiety-like behavior than CC mice. **(A)** Time spent in the open arms of the arena, **(B)** time spent in the outer (farthest from the center) 50% of the open arms, and **(C)** total distance traveled in the arena. * represents a significant main effect of treatment ($p<0.05$). Data are represented as means ± SEM (n=10-22).

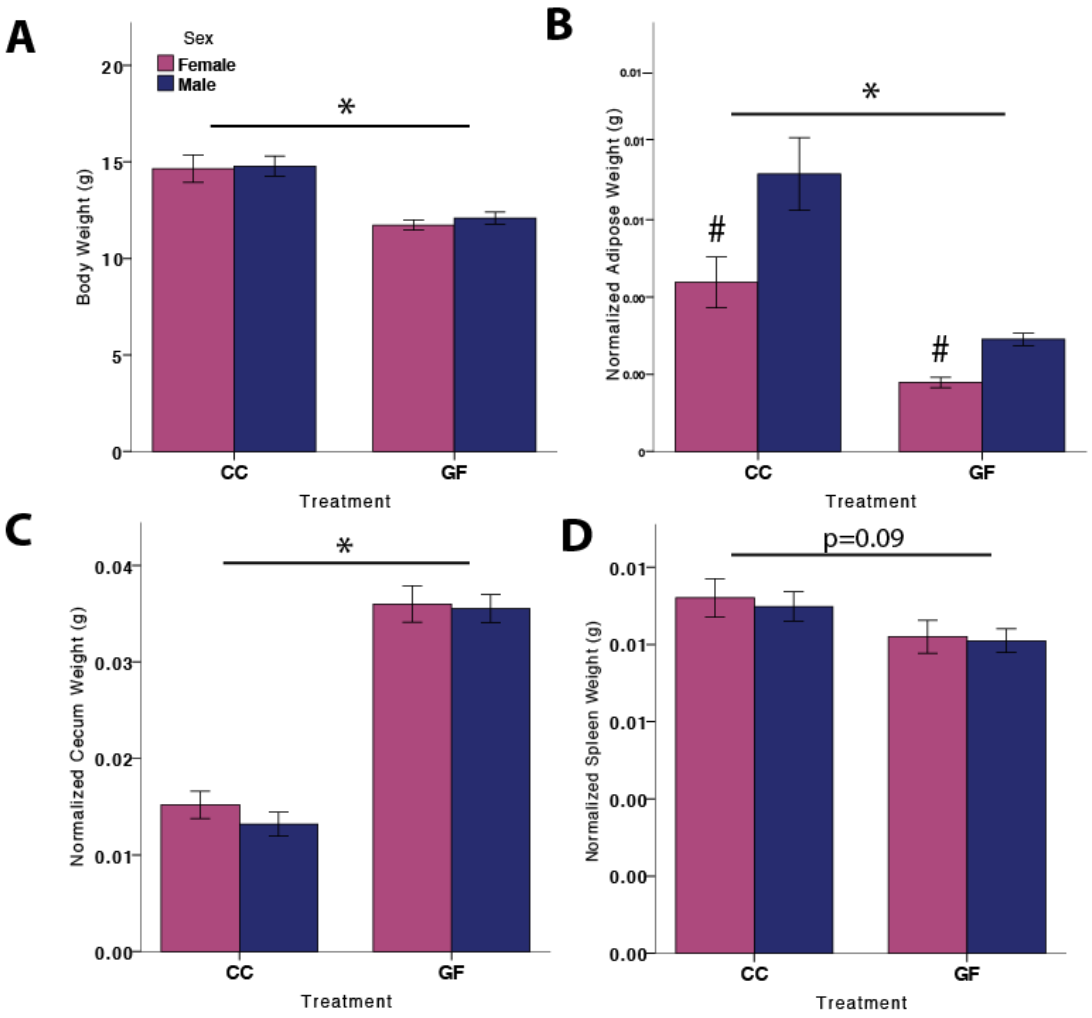

Figure 2.10. Weanling-aged GF mice show adult-typical GF physiology.

(A) Total body weight of mice, **(B)** normalized gonadal fat pad weight, **(C)** normalized cecum weight, and **(D)** normalized spleen weight. * represents a significant main effect of treatment ($p<0.05$). Trends toward significance are represented with the p-value. Data are represented as means ± SEM (n=10-22).

3 KNOCKOUT OF TOLL-LIKE RECEPTOR 5 RESULTS IN AN ANXIOGENIC PHENOTYPE ASSOCIATED WITH CHANGES IN NEURAL VASOPRESSIN THROUGH A MICROBIOTA-INDEPENDENT PATHWAY

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

3.1 Abstract

Inflammation contributes to the strong comorbidity between psychiatric and gastrointestinal disorders, but the exact mechanisms underlying this connection are still unclear. To investigate this, we are using a model of chronic intestinal inflammation and metabolic syndrome, induced by the knockout of toll-like receptor 5 (T5KO), to determine: i) the effects of chronic inflammation and metabolic disruption on behavior, ii) the neural circuitry that may contribute to the changes in behavior, and iii) the role microbiota may play in this pathway. First, we phenotyped adult male and female T5KO and wild-type (WT) mice in anxiety-like, depressive-like, and social behavior assays. Then we quantified neural OXT and vasopressin (AVP) immunoreactivity. We found that T5KO mice have an anxiogenic and depressive-like phenotype that was associated with changes in AVP in projection sites of the paraventricular nucleus of the hypothalamus (PVN) and suprachiasmatic nucleus (SCN). To test whether the behavioral phenotype is due to the actions of microbiota, we colonized GF mice at weaning with microbiota from T5KO or WT mice, then behaviorally analyzed the offspring of these microbiota-treated mice. We found that while adult T5KO-gavaged (T5KO-g) mice show evidence of intestinal inflammation similar to T5KO mice, they did not show behavioral changes. Microbiota transplantation was not sufficient to induce

behavioral or physiological changes in juvenile mice. Thus, intestinal inflammation promotes anxiety-like and depressive-like behavior, partially due to changes in AVP neural circuitry, through a microbiota-independent pathway.

3.2 Introduction

In the developed world, there has been a reduction in infectious diseases with a simultaneous increase in chronic inflammatory disorders (Powell et al., 2017). While inflammatory disorders can affect all areas of the body, particular attention has been paid to those gastrointestinal and psychiatric in nature, such as irritable bowel syndrome or anxiety and mood disorders. Recent evidence of the considerable cross-talk between the gut and the brain may explain the high levels of comorbidity between intestinal inflammation and psychiatric disorders (Dinan and Cryan, 2016). In fact, signals from the brain can influence gut function, and vice versa. To add to the complexity, the composition of gut microbiota can have an effect on both gut and brain function, due to the myriad of neural, endocrine, metabolic, and immune signals they send to the rest of the body (reviewed in Martin et al., 2018). Thus, understanding the signaling pathways of the microbiota-gut-brain axis, particularly in the context of inflammation, can have significant implications for human mental health.

There are a number of models of intestinal inflammation that are used to probe how inflammation affects the brain. Dextran sodium sulfate is commonly used to elicit colitis, severe inflammation of the colon, but this provides a model of acute, not chronic, inflammation (Emge et al., 2016). Others use dietary changes, such as diets containing high fat concentrations or dietary additives like emulsifiers or artificial sweeteners, to

illicit inflammation (Chassaing et al., 2015; de Sousa Rodrigues et al., 2017), but these models may have downstream effects on the body that are still unknown. Another way is through the acute or chronic administration of lipopolysaccharide (LPS), a protein found on the outer membrane of pathogenic, Gram-negative bacteria, which increases gut inflammation and induces sickness behavior. Recently, our lab found that orally-gavaged LPS can increase anxiety-like and repetitive behaviors through sexually dimorphic mechanisms (Fields et al., 2018a). Genetic models are also used to look at chronic intestinal inflammation, like interleukin (IL)-10 or MUC2 (a major glycoprotein in colonic mucus) knockouts (Leon et al., 1998; Kumar et al., 2017). To best understand the gut-brain communication pathways in a chronic intestinal inflammatory and disrupted metabolic state, a Toll-like receptor 5 (TLR5) knockout model should be used for the reasons described below.

Toll-like receptors (TLRs) are pattern-recognition receptors that respond to conserved protein patterns on the surfaces of bacterial membranes. TLR5 specifically responds to flagellin, a component of flagellum on motile bacteria, and are primarily located along the basolateral surface of the gut epithelium (Gewirtz et al., 2001; Hayashi et al., 2001). T5KO mice have a larger intestinal bacterial load and more epithelial-adherent bacteria, as well as a thinner protective layer of mucus along the gut epithelium (Carvalho et al., 2012b). It is hypothesized that these changes to the gut epithelium lead to invasion of bacteria into the peritoneum, leading to a robust immune response to manage the intrusion (Carvalho et al., 2012; Etienne-Mesmin et al., 2016). Knockout of TLR5 results in chronic intestinal inflammation, as characterized by elevated levels of the systemic pro-inflammatory cytokines interleukin-1β (IL-1β) and

tumor necrosis factor-α (TNF-α), and other signs of inflammation like elevated lipocalin-2, mild splenomegaly, and shorter and heavier colons (Carvalho et al., 2011; Carvalho et al., 2012). T5KO mice also show symptoms of metabolic syndrome, including obesity due to larger gonadal adipose deposits, insulin resistance and increased serum cholesterol levels (Vijay-Kumar et al., 2010). These symptoms take many weeks to develop (Vijay-Kumar et al., 2010). Furthermore, while there are only some species level differences between the microbiota compositions of T5KO and wild-type (WT) mice, the T5KO phenotype is microbiota-dependent (Vijay-Kumar et al., 2010). Transplantation of T5KO microbiota into germ-free, WT mice induced the same metabolic syndrome and intestinal inflammation characteristics as knockout of TLR5.

Inflammation contributes to the pathologies of anxiety and mood disorders; therefore, it is possible that T5KO mice will have a behavioral phenotype indicative of mild sickness behavior, including increased anxiety-like and depressive-like behaviors and decreased social motivation (Dantzer et al., 2008). The neuropeptides OXT and AVP are sensitive to peripheral immune signals and have roles in mediating anxiety and social behaviors (reviewed in Bredewold & Veenema, 2018; Caldwell, 2017; Jurek & Neumann, 2018). Thus, we hypothesize that intestinal inflammation increases anxiety- and depressive-like behaviors through affecting OXT and AVP.

These factors led us to question 1) does knockout of TLR5 affect the behavior of the mouse, 2) are the neuropeptides OXT and AVP altered in T5KO mice and 3) are any behavioral changes microbiota-dependent? To test these questions, we first behaviorally phenotyped T5KO mice and characterized OXT and AVP expression in limbic brain regions. Then, we tested the offspring of T5KO microbiota-transplanted GF

mice to determine if T5KO microbiota was sufficient to recapitulate the T5KO behavioral phenotype. We found that while T5KO mice had an anxiogenic and depressive behavioral phenotype associated with changes to AVP, T5KO microbiota alone was not sufficient to produce this phenotype in WT mice.

3.3 Materials and Methods

3.3.1 Experiment 1

3.3.1.1 Animals

Adult male and female T5KO and WT C57Bl/6 mice were obtained from an in-house breeding colony at Georgia State University. Mice were housed in ventilated transparent Optimouse cages (35.6 x 48.5 x 21.8cm) lined with Bed-O-Cobs® bedding, with nestlets and shelters for enrichment. Animals were kept on a 12h:12h light:dark cycle (lights off at 1900 EST) and ambient temperature was kept at 23°C. Food (Purina rodent chow no. 5001) and water were available *ad libitum*. Animals were weaned at postnatal day 21 (P21) and housed with littermates of the same sex and genotype group. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the Georgia State University.

3.3.1.2 Behavioral Testing Schedule

Behavioral tests were conducted in the following order: Open Field Test, Elevated Plus Maze, Light/Dark Box, Marble Burying Task, Three-Chamber Sociability Task, and Forced Swim Test. Tests were performed 3-4 days apart, with the exception of the sociability and swim tests, which only had 2 days between testing. Behavioral testing occurred within the last 4 hours of the light phase of the light:dark cycle with

overhead lights as illumination. Animals were allowed to habituate to the testing room for 1 hour prior to testing. Apparatuses were cleaned with 70% ethanol between animals in a testing session to remove the scent of previously-tested mice and Vimoba solution (chlorine dioxide, Quip Laboratories, Wilmington, DE) to sterilize the arena at the end of testing sessions. An experimenter blind to genotype conditions scored all behavioral tests.

Two different cohorts were used in the three-chamber sociability test. The first cohort used only WT stimulus animals for both T5KO and WT experimental mice. The second cohort used a matched genotype stimulus mouse, such that T5KO mice were exposed to an unfamiliar T5KO mouse, and WT mice exposed to an unfamiliar WT mouse.

3.3.2 Experiment 2

3.3.2.1 Animals

Germ-free Swiss-Webster mice were obtained from our breeding program at Georgia State University and maintained in a Park Biosciences isolator as previously described (Chassaing et al., 2015). Mice were removed from isolator at weaning (P21) and were orally administered with 200 µL of fecal suspension from an age-matched designated donor. The fecal suspensions were sourced from wild-type or T5KO C57BL/6 mouse donors. Transplanted mice were then housed in four breeding groups (one male, two females) in isolated ventilated cages, Isocages (Techniplast, West Chester, Pennsylvania, USA), and fed autoclaved Purina Rodent Chow # 5021, as previously described (Chassaing & Gewirtz, 2018). Animals were kept on a 12h:12h light:dark cycle (lights off at 1900 EST) and ambient temperature was kept at

23°C. Breeding groups were removed from Isocages once reaching adulthood (P60), once microbiota composition had stabilized, and kept in conventional animal housing as described in Experiment 1.

All mice were weighed weekly and dams were examined for signs of pregnancy. Pregnant females were removed from the breeding groups and housed singly until the pups were weaned at P21. Weaned mice were housed with littermates of the same sex and treatment group. Mice were fed Purina Rodent Chow #5021 while in breeding groups and until weaning, at which point their food was changed to Purina Rodent Chow #5001. All cage changes occurred in a biosafety cabinet to prevent microbiota cross-contamination and new gloves were used between openings of cages of each microbiota treatment during weighing and behavioral testing.

3.3.2.2 Behavioral Testing Schedule

All mice underwent behavioral testing at weaning, with half of the litters assayed on the Open Field Test and Elevated Zero Maze on P22 and Social Interaction Test and Tail Suspension Test on P24, with the other half tested on P23 and P25. Behavior was conducted in the same manner as in Experiment 1. Half of the litters were allowed to grow into adulthood, and the other half were sacrificed at P29 to determine the effects T5KO microbiota have on development.

Adult (12-14 weeks of age) mice were tested in the Open Field Test, Elevated Zero Maze, Light/Dark Box, Marble Burying Task, Three-Chamber Sociability Test, and Tail Suspension Test as described above.

The elevated zero test was chosen in place of the elevated plus test due to its ability to be repeated without affecting the results of the test (Tucker and McCabe,

2017). The tail suspension test was chosen in place of the forced swim test because the T5KO mice in experiment 1 did not move around the apparatuses as much as the WT mice, and we wanted to minimize the chance that changes in depressive-like behavior were just due to lack of movement. Finally, we used a social interaction paradigm only for the weanling-aged mice due to the lack of age-matched stimulus mice.

3.3.3 Descriptions of Behavioral Assays

3.3.3.1 Open Field Test (OFT)

Locomotor behavior was assessed in a Plexiglas arena (43.2 cm W X 43.2 L X 30.5 cm H; Med Associates Inc., St. Albans, VT) containing 2 infrared transmitter strips (16 beams each) at the bottom of the arena in the X and Y planes, dividing the arena into 256 squares. Another infrared transmitter strip was located 14 cm from the bottom of the arena to assess behavior in the Z plane. The center of the apparatus was defined as the center 8 beams in both the X and Y planes. Each mouse was placed into the arena against the wall closest to the experimenter and allowed to freely explore the apparatus for 10 minutes. The total distance traveled, and time spent in the center of the arena were automatically calculated by Activity Monitor (Med Associates, Inc.) on a computer connected to the open field arenas.

3.3.3.2 Elevated Plus Maze (EPM)

A standard mouse elevated plus maze (EPM) was used, with 2 open arms and 2 closed arms. The arms were 10 cm W x 50 cm L, connected by a 10 cm X 10 cm center square. Closed arms had a wall height of 40 cm, and the maze was elevated 50 cm from the floor. At the beginning of the test, mice were placed in the center square of

the arena and allowed to freely explore for 5 min. Video trials were recorded from a digital camera mounted above the apparatus that was connected by USB to a computer. The time spent in open arms and total distance traveled were quantified by AnyMaze version 4.96 (Stoelting, Co., Wood Dale, IL).

3.3.3.3 Elevated Zero Maze (EZM)

A standard mouse elevated zero maze (EZM) was used. The apparatus consisted of a 5.5 cm wide circular platform of internal diameter 35 cm, raised 50 cm off the ground, with two equally spaced enclosed compartments covering half the platform. At the beginning of the test, mice were placed at the intersection of the enclosed and open sections of the arena and allowed to freely explore for 5 min. Video trials were recorded from a digital camera mounted above the apparatus that was connected by USB to a computer. The time spent in open and closed zones of the apparatus was quantified and broken down into time spent walking, immobile, rearing, and grooming. Time spent in stretch-attend posture, defined as elongated body posture to investigate the open zone with at least 50% of the body in the closed zone (Grant and Mackintosh, 1963). These behaviors were scored by a researcher blind to treatment condition using AnyMaze version 4.96 (Stoelting, Co., Wood Dale, IL).

3.3.3.4 Light/Dark (L/D) Box

An acrylic box (14.5 cm W X 30 cm L X 14 cm H) was split into a light chamber (20 cm long) made out of white acrylic, and a dark chamber (10 cm long) made out of opaque black acrylic and covered. An opaque insert with a 5 cm W X 5 cm H opening separated the two chambers, in order to allow the animals to travel freely between the 2 compartments. Mice were placed in the light chamber by the edge farthest away from

the dark compartment and were allowed to investigate for 5 min. Video trials were recorded from a digital camera mounted above the apparatus that was connected by USB to a computer. The number of entries into the light chamber and the total time spent in the light were quantified by AnyMaze version 4.96 (Stoelting, Co., Wood Dale, IL).

3.3.3.5 Marble Burying Test

A Plexiglas arena (24cm W X 46 cm L) was filled with 4 cm of Alpha-dri bedding (Shepherd Specialty Paper, Fibercore, Cleveland, OH, USA). Mice were placed into the arena for a 5-minute habituation period, then removed in order to place 20 marbles (17mm) in an evenly spaced, 4x5 grid on top of the bedding. Mice were returned to the center of the arena and their behavior was video recorded for 10 min. The number of marbles buried during this period, defined as being half or more covered by bedding, time spent grooming, and total time spent digging were quantified using the Observer XT 11.5 (Noldus Information Technology, Wageningen, The Netherlands). Distance traveled in the arena was calculated by AnyMaze version 4.96 (Stoelting, Co., Wood Dale, IL).

3.3.3.6 Three-Chamber Sociability Test (TCT)

A polycarbonate chamber (24cm W X 74cm L X 24 cm H) was split equally into 3 equal sized chambers, with a 9 cm W opening between each to allow movement between them. At either end of the apparatus was an opening (9cm W X 10 cm H) to allow access to the stimulus cages. The stimulus cages were also made of polycarbonate (10cm W X 10 cm L X 10 cm H) with a 10 X 10 grid of 0.5cm diameter holes to allow transmission of odor and visual cues, while limiting physical contact to

whisking or nose contact. The three-chamber sociability task was conducted in three phases, as described below.

3.3.3.6.1 Habituation

Animals were placed into the center of the apparatus, lined with absorbent lab paper, with empty stimulus cages in order to habituate the animals to the apparatus. Animals were allowed to explore the apparatus for 5 minutes and were recorded using an overhead camera.

3.3.3.6.2 Social Investigation

After the habituation period, the experimental animals were removed from the apparatus and an unfamiliar, sex-matched C57Bl/6 mouse was placed in one of the stimulus chambers. A novel object was placed in an identical stimulus chamber, and both stimulus chambers were placed against the side chambers on opposite sides of the apparatus. The positioning of the mouse chamber and novel object chamber were alternated for each test, in order to avoid chamber preferences. The experimental animal was placed back into the apparatus and freely allowed to explore for 10 minutes. The time spent in each chamber and number of chamber entries were quantified by Anymaze, and the amount of time spent actively investigating each chamber was hand scored by an investigator blind to the experimental groups.

3.3.3.6.3 Social Preference

A different cohort of mice were used in the social preference task. Due to differing behavioral results from the genotype of mouse used as the stimulus animal during the investigation phase, we tested whether the experimental mice had a preference for investigating a T5KO or WT mouse. These mice were allowed a 5-

minute habituation to the apparatus, then they were removed and an unfamiliar, sex-matched mouse of each genotype (WT or T5KO) was placed into the stimulus chamber to allow the experimental mouse to choose between investigating a T5KO or WT mouse. The experimental mouse (either T5KO or WT) was allowed to investigate the arena freely for 10 minutes. The time spent in each chamber and the amount of time spent actively investigating each stimulus mouse were by an investigator blind to experimental groups using Observer XT 11.5 (Noldus Information Technology, Wageningen, The Netherlands).

3.3.3.7 Social Interaction

A Plexiglas arena (24cm W X 46 cm L) was filled with 2 cm of Alpha-dri bedding (Shepherd Specialty Paper, Fibercore, Cleveland, OH, USA). Two mice from the same litter (and therefore the same treatment) were placed into the arena and video recorded for 10 minutes. Time spent walking, immobile, grooming, allogrooming, rearing, digging, and investigating the other mouse were scored using Observer XT 11.5 (Noldus Information Technology, Wageningen, The Netherlands).

3.3.3.8 Forced Swim Test (FST)

Mice were placed into a Plexiglas cylinder containing 3L of water at 28°C±2° for 5 min. At the end of the test, mice were removed from the cylinder and gently dried with a clean towel. The duration of mobility, defined as attempts to escape the cylinder and active swimming, and the duration of immobility, defined as absence of movement or small movement of posterior paws used for floatation only, were scored using Observer XT by an investigator blind to experimental groups.

3.3.3.9 Tail Suspension Test (TST)

Animals were suspended by their tails by a strip of tape (~15 cm) attached to an overhang and recorded for 5 minutes. A pipette tip was placed on their tail before the tape to prevent the mouse from holding onto their tail during the test. Time spent struggling or hanging was analyzed using Noldus Observer.

3.3.4 Euthanasia and Tissue Collections

After completion of behavioral testing in both experiments, mice were deeply anesthetized using isoflurane (5%v/v). Blood was collected by retrobulbar intraorbital capillary plexus. Hemolysis-free serum was collected by centrifugation of blood using serum-separator tubes (Becton Dickinson, Franklin Lakes, NJ). Following blood collection, mice were euthanized by cervical dislocation. The weight and length of the colon and weights of the spleen, liver, and perigonadal adipose fat depot were recorded. Feces were collected for microbiota analysis. 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). 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.

3.3.5 Immunohistochemistry

Free-floating sections were rinsed five 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 five times in TBS, sections were places for 30 min in 0.1 M glycine in TBS, rinsed again, and placed into block solution (10% normal goat serum (NGS), 0.4%

Triton-X and 1% H2O2 in TBS) for 30 min. Sections were then incubated overnight in one of the following primary antibodies: anti-AVP (Bachem; 1:32000) or anti-OXT (Peninsula Labs; 1:32000); all dilutions in TBS with 2% NGS and 0.4% Triton-X. The next day, sections were rinsed five times in TBS containing 1% NGS and 0.02% Triton-X and incubated in biotinylated secondary antiserum [goat anti-rabbit for AVP immunoreactivity; goat anti-guinea pig for OXT (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 coverslipped with Permount.

3.3.6 Colonic Myeloperoxidase Assay

Colonic myeloperoxidase, a marker for neutrophils, was analyzed as previously described (Chassaing et al., 2015). In brief, tissue was washed in PBS and homogenized in 0.5% hexadecyltrimethylammonium bromide (Sigma, St. Louis, Missouri) in 50mM PBS (pH 6.0), freeze-thawed three times, sonicated and centrifuged. Supernatant was analyzed for myeloperoxidase by adding dianisidine dihydrochloride (Sigma, St. Louis, Missouri) and H_2O_2 and measuring the optical density at 450nm. Human neutrophil myeloperoxidase (Sigma, St. Louis, Missouri) was used as a standard.

3.3.7 LCN2 ELISA

Serum supernatant was analyzed for Lcn-2 using Duoset murine Lcn-2 ELISA kit (R&D Systems, Minneapolis, Minnesota) as previously described (Chassaing et al., 2015). Optical density was measured at 450nm.

3.3.8 Image Analysis

Matched sections based on the Mouse Brain Axis (Franklin and Paxinos, 2008) and location of staining for each mouse were imaged using a Zeiss Axio Imager M2 microscope connected to an ORCA-R2 CCD digital camera (Hamamatsu Photonics). Brain regions were selected from each of the three neuropeptide source and projection pathways: the PVN/SON pathway, the BNST pathway, and SCN pathway (as described in Rood & De Vries, 2011). The PVN/SON pathway includes the PVN. The BNST-MA pathway includes the lateral habenula (LHb), ventral lateral septum (LS), and mediodorsal nucleus of the thalamus (MD). The SCN pathway includes the SCN, subparaventricular zone (SPZ), paraventricular nucleus of the thalamus (PVT), and the dorsomedial nucleus of the hypothalamus (DMH). 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.

3.3.9 Statistical analyses

Data were analyzed and visualized using IBM SPSS Statistics Version 21 (IBM). Anxiety-like and social behaviors, as well as body and organ weights, were analyzed by a two-way ANOVA with treatment and sex as the factors, followed by

Bonferroni post hoc analyses. Data were analyzed by MANOVA followed by discriminant analysis to reveal patterns in the behavioral phenotype, as previously described (Fields et al., 2018a).

3.4 Results

3.4.1 Experiment 1: Behavioral and neural phenotyping of T5KO mice

3.4.1.1 T5KO mice show symptoms of metabolic syndrome and low-grade inflammation

As expected, T5KO showed morphological signs of metabolic syndrome, including increased body weight (Figure 3.1A; $F(1, 67)= 14.61$, $p<0.001$) and increased gonadal fat pad weight (Figure 3.1B; $F(1, 72)= 24.126$, $p<0.001$). Males were heavier ($F(1, 67)= 122.004$, $p<0.001$) and had larger fat pads ($F(1, 72)=52.585$, $p<0.001$) than females. T5KO mice showed morphological signs of low-grade inflammation, including shorter (Figure 3.1C, $F(1, 72)= 57.6$, $p<0.001$) and heavier (Figure 3.1D; $F(1, 72)= 25.384$, $p<0.001$) colons as well as mild splenomegaly (Figure 3.1E; $F(1, 72)=23.491$, $p<0.001$). Females in both groups had heavier spleens than males ($F(1, 72)= 8.844$, $p=0.004$). Levels of Lcn-2 increased in female T5KO mice and decreased in male T5KO mice compared to their WT counterparts (Figure 3.1F; sex by treatment interaction, $F(1, 53)= 35.242$, $p<0.001$). There was no difference between genotypes in MPO levels, indicating that T5KO mice are not colitic (Figure 3.1G; $p>0.05$).

3.4.1.2 T5KO mice have an anxiogenic and depressive phenotype

Knockout of TLR5 in females resulted in decreased time spent in the center of the open field arena (Figure 3.2A, main effect of treatment, $F(1, 72)= 5.896$, $p=0.01$; main effect of sex, $F(1, 72)= 4.585$, $p=0.036$). This behavior may be partially due to the

fact that T5KO mice traveled less distance in the arena (Figure 3.2B, main effect of treatment, $F(1, 72)= 40.246$, $p<0.001$), suggesting that T5KO mice were not exploring the arena as much as the WT mice. When these data were analyzed using distance traveled as a covariate, there was a trend towards an interaction between sex and treatment ($F(1, 72)=3.644$, $p=0.06$), but there was no main effect of genotype on time spent in the center of the OFT ($p>0.05$).

Similar to their behavior in the open field, female T5KO mice spent the least amount of time in the open arms of the EPM (Figure 3.2C, main effect of sex, $F(1, 72)= 4.025$, $p=0.049$). However, T5KO mice overall did not show differences in open arm time compared to WT mice, which led to a trend towards an interaction of treatment and sex ($F(1, 72)= 3.682$, $p=0.059$). Again, T5KO mice traveled less in the EPM than the WT mice (Figure 3.2D, $F(1, 72)=4.751$, $p=0.033$), which may partially explain the difference in the female T5KO behavior. When we use distance traveled as a covariate, there was no effect of genotype on time spent in the open arms of the elevated plus maze ($p>0.05$).

T5KO mice spent half the time the WT mice did in the light chamber of the light/dark box (Figure 3.2E, $F(1, 71)= 35.474$, $p<0.001$). We did not observe any sex differences in time in the light zone ($p>0.05$). Because the dark chamber is covered from camera view, we used the number of light chamber entries as a proxy of distance traveled in the apparatus. There was no difference between genotype in light zone entries (Figure 3.2F, $p>0.05$). There was still a significant effect of genotype when the number of entries into the light zone was used as a covariate ($F(1, 71)=33.325$, $p<0.001$).

T5KO mice spent twice as long floating in the FST than WT mice (Figure 3.2G, $F(1, 72)= 46.846$, $p<0.001$), typically indicative of a depressive-like phenotype. However, this effect may be confounded by the fact that T5KO mice were less mobile in the other behavioral assays and that T5KO had more fat tissue. Knockout of TLR5 had more of an effect on the males, resulting in a trend towards a sex difference (main effect of sex, $F(1, 72)= 5.269$, $p=0.025$, independent t-test, $p=0.059$). T5KO mice also had a shorter latency to begin floating than WT mice (Figure 3.2H, $F(1, 40)= 14.886$, $p<0.001$).

The marble burying test is used as a measure of compulsive behavior (Angoa-Pérez et al., 2013). There were no differences between T5KO and WT mice in number of marbles buried in the arena (Figure 3.3A, $p>0.05$), but males buried more marbles than females ($F(1, 72)= 18.292$, $p<0.001$). Males spent more time digging in the bedding of the arena than the females (Figure 3.3B, $F(1, 72)= 16.849$, $p<0.001$). While there were no differences between T5KO and WT mice in digging behavior, T5KO mice spent more time grooming in the arena than WT mice (Figure 3.3C, $F(1, 71)= 16.589$, $p<0.001$). T5KO mice also traveled less distance than WT mice (Figure 3.3D, $F(1, 71)= 42.255$, $p<0.001$). When distance traveled was used as a covariate in the ANCOVA, we found that there was a trend towards an effect of genotype in the number of marbles buried ($F(1, 71)= 3.099$, $p=0.083$).

3.4.1.3 T5KO mice were more social only with WT stimulus mice

Three different cohorts of mice were used in the three-chamber sociability test: one with all WT stimulus mice, one with matched genotype stimulus mice, and one where a choice of T5KO or WT mouse was given. T5KO mice investigated both the

stimulus WT mouse (Figure 3.4A, $F(1, 40)= 25.237$, $p<0.001$) and novel object (Figure 3.4B, $F(1, 40)= 19.81$, $p<0.001$) more than the WT mice. When the stimulus mouse was of matched genotype, there was a trend towards an increase in social interaction in the T5KO mice (Figure 3.4C, $F(1, 31)= 3.98$, $p=0.056$), and no difference in time investigating the novel object (Figure 3.4D, $p>0.05$). When the distances traveled from the first two cohorts were combined, T5KO mice traveled less in the arena than WT mice (Figure 3.4E, $F(1, 72)= 63.412$, $p<0.001$). WT females traveled more than WT males, and this sex difference was lost in the T5KO mice ($F(1, 72)= 7.5$, $p=0.008$). Finally, when distance was used as a covariate, there was a main effect of genotype in time investigating the mouse in the first (WT only) cohort ($F(1, 40)= 12.937$, $p=0.001$, but there was no effect of genotype when the stimulus animals were of matched genotypes ($p>0.05$).

Due to the fact that T5KO mice were more social when presented with a WT stimulus mouse than one of their own genotype, we decided to test the preference of T5KO and WT mice for a mouse of their same or different genotype. WT females, T5KO females and T5KO males preferred WT stimulus mice, as evidenced by a positive preference score (Figure 3.4F, sex by treatment interaction, $F(1, 37)= 4.562$, $p=0.04$). WT males, however, greatly preferred T5KO mice. Thus, with the exception of WT females, mice prefer to investigate the different genotype stimulus.

3.4.1.4 Knockout of TLR5 increased AVP in PVN and SCN projection sites

In the PVN, T5KO mice had greater levels of AVP-ir than WT mice (Figure 3.5A). This difference was due to greater immunoreactivity in the T5KO males, resulting in main effects of both genotype ($F(1, 35)=9.353$, $p=0.004$) and sex ($F(1,$

35)=14.357, p=0.001). A similar pattern was seen in the DMH, PVT, and SPZ, projection sites of the SCN. In the DMH, T5KO mice had higher levels of AVP-ir (Figure 3.5B, $F(1, 35)= 10.238$, p=0.003), due to greater immunoreactivity in the T5KO males ($F(1, 35)= 5.633$, p=0.024), but this did not reach significance in the PVT (Figure 3.5C; trend towards main effect of genotype, $F(1,35)=3.037$, p=0.091; trend towards main effect of sex, $F(1,35)= 3.822$, p=0.059), nor the SPZ (Figure 3.5D; trend towards main effect of genotype, $F(1, 35)= 3.107$, p=0.088, main effect of sex, $F(1, 35)= 7.502$, p=0.01).

In the SCN, T5KO males had greater AVP-ir compared to the other groups (Figure 3.5E; $F(1, 35)= 4.213$, p=0.048), resulting in overall greater immunoreactivity in the T5KO mice, but this trend did not quite meet significance ($F(1, 35)= 3.573$, p=0.068).

In the LHb, the typical sex difference in this region, where males have higher AVP-ir than females, was greater in the T5KO mice than WT mice, resulting in a sex by treatment interaction (Figure 3.5F; $F(1, 35)= 17.93$, p<0.001), such that AVP-ir decreased from WT to T5KO females and increased in the males. The same pattern was seen in the ventral LS, albeit with a lesser increase in the sex difference (Figure 3.5H; sex by treatment interaction, $F(1, 35)=4.741$, p=0.037). There were no genotypic effects on AVP-ir in the MD, but a large sex difference was observed (Figure 3.5G; $F(1, 34)= 57.933$, p<0.001), where males had higher immunoreactivity than the females, in accordance with the literature (Rood et al., 2013).

3.4.1.5 T5KO increased OXT in the BNSTmv and AH

T5KO mice showed higher levels of OXT-ir in the medial ventral BNST (Figure 3.6A; $F(1, 37)=5.699$, $p=0.023$). There were no differences between the sexes in OXT-ir ($p>0.05$).

In the anterior hypothalamus, there was a sex by genotype interaction in the number of OXT positive cells (Figure 3.6B; $F(1, 38)= 5.49$, $p=0.025$). This interaction was due to the increase in OXT-ir positive cells in the male T5KO mice compared to male WT and the abolishment of the sex difference in the T5KO mice. This pattern was only seen in the number of OXT-positive cells, not the quantification of OXT-ir. Female mice in both genotypes had higher levels of OXT-ir compared to the males ($F(1, 38)=6.153$, $p=0.018$, data not shown).

A sex difference emerged in the DMH in T5KO mice compared to WT mice, where female T5KO mice had greater OXT-ir than the males (Figure 3.6C; $F(1, 38)=4.323$, $p=0.045$). In the SPZ, female WT mice had higher levels of OXT-ir than the WT males, but there was no sex difference in the T5KO mice (Figure 3.6D; $F(1, 37)=3.233$, $p=0.081$).

Females showed a trend towards greater OXT-ir levels in the SON than males, but this did not reach significance (Figure 3.6E; $F(1, 38)= 3.573$, $p= 0.067$). There were no differences in the number of OXT positive cells between genotypes or sexes ($p>0.05$; data not shown).

There was no effect of genotype or sex on OXT-ir or OXT positive cells in the PVN (Figure 3.6F; $p>0.05$). There were also no effects of sex or genotype on OXT immunoreactivity in the ventral LS, BNST, MnPO, or PVT.

3.4.1.6 Multivariate statistics separated groups by sex and genotype for both behavioral profile and neuropeptide expression

We wanted to see if the behavioral changes we saw in the various assays resulted in an aggregate behavioral phenotype. To do this, we used discriminant analysis to determine the contribution of each behavioral measured outcome to the overall phenotype (Figure 3.7A). When subjects were designated to four groups based on sex and genotype, discriminant analysis revealed three canonical functions that maximize group separation along these factors. Function 1 explains 79.9% of the variance (canonical R^2= 0.856), function 2 explains 18% of the variance (canonical R^2= 0.617), and function 3 explains 2.1% of the variance (canonical R^2= 0.259). Collectively, these discriminant functions significantly differentiated the sex and genotype groups (Λ=0.154, $X^2(24)$= 119.536, p<0.001). Table 1 reveals the correlations between behavioral measures and the discriminant functions. In this case, function 1 primarily separated T5KO mice from WT mice and was driven by time spent floating in the TST, distance traveled in the OFT, time spent investigating the mouse in the TCT, and time spent self-grooming in the marble burying test. Function 2, which primarily separated males from females, was driven by the number of marbles buried, time in light zone of the L/D box, and time in the center of the OFT. This pattern is similar to that seen in the individual graphs, where the factors underlying function 1 had larger effects between genotypes, whereas the outcomes in function 2 showed sex differences.

The same procedure was done for neuropeptide expression (Figure 3.7B). Function 1 explained 74.8% of the variance (canonical R^2=0.921), function 2

explained 22.7% of the variance (canonical R^2=0.793), and function 3 explained 2.5% of the variance (canonical R^2=0.398). Collectively, these functions differentiated between sex and genotype as well (Λ=0.047, $X^2(33)$= 74.798, p<0.001). Function 1 separated the cases mostly by sex, so it was unsurprising that this was driven by AVP-ir in the LHb and MD, as both of these regions had distinct sex differences. Function 2, which split the cases by genotype, was driven by AVP-ir in the DMH, PVN, and PVT, and OXT-ir in the DMH. These results suggest that AVP is the main neuropeptide contributing to the separation between sex and genotype.

3.4.2 Experiment 2: T5KO and WT microbiota transplantation to WT mice

In this experiment, we examined the offspring of microbiota-transplanted GF mice at weaning and adulthood to see if the behavioral phenotype described above was due to microbiota composition. The offspring were used in behavioral studies because we wanted the experimental mice to have had T5KO or WT microbiota throughout their lifetimes. However, we predicted that there would be no effect of T5KO microbiota transplantation in the weanling-aged mice, because the physiological effects of T5KO and T5KO microbiota transplantation take many weeks to develop (Vijay-Kumar et al., 2010).

3.4.2.1 T5KO microbiota have little effect on physiology in juvenile mice

WT-g mice had a higher body weight than T5KO-g mice at P29 (F(1, 56)= 12.719, p=0.001) and males were heavier than females (Figure 3.8A; F(1, 56)= 7.673, p=0.008). This was due in part to smaller adipose deposits in the T5KO-g mice (Figure 3.8B; F(1, 56)=83.444, p<0.001). A sex difference appeared in the T5KO-g mice, where males had heavier adipose pads than females (F(1, 56)= 8.742, p=0.005). There was

no difference between groups in colon weight (Figure 3.8C; p>0.05), but T5KO males had longer colons than T5KO females (Figure 3.8D; $F(1, 56)=6.213$, p=0.016). Males had heavier ceca than females (Figure 3.8E; $F(1, 56)= 18.002$, p<0.001). Spleen weight decreased in T5KO males compared to WT males (Figure 3.8F; sex by treatment interaction, $F(1, 56)=4.947$, p=0.03), and males had heavier spleens than females.

3.4.2.2 T5KO microbiota had no effect on anxiety-, depressive-like, or social behaviors at weaning

There was no effect of sex or treatment group on time spent in the center of the apparatus nor distance traveled in the arena in the OFT (Figures 3.9A and 3.9B; p>0.05), time in the open zones of the EZM (Figure 3.9C; p>0.05), nor time spent hanging in the TST (Figure 3.9D; p>0.05).

Treatment with T5KO microbiota had the biggest impact on movement in the social interaction test. For example, WT-g mice spent more time moving than T5KO-g (Figure 3.10A; significant treatment by sex interaction; $F(1, 99)=6.682$, p=0.011), particularly the females, and spent less time immobile (Figure 3.10B; trend towards main effect of treatment, $F(1, 83)= 3.311$, p=0.073). In addition, a number of sex differences in behavior were seen. Males spent more time in the arena investigating the other mouse (Figure 3.10C; $F(1, 95)= 6.469$, p=0.013) and grooming themselves (Figure 3.10D; $F(1, 97)= 11.4$, p=0.001), whereas females spent more time digging (Figure 3.10E; $F(1, 59)= 5.255$, p=0.026) and rearing (Figure 3.10F; trend towards main effect of sex, $F(1, 75)= 3.717$, p=0.058).

3.4.2.3 T5KO microbiota is sufficient to recapitulate T5KO physiological phenotype in adult mice

Adult T5KO-g mice had characteristics of metabolic syndrome and chronic intestinal inflammation. T5KO-g mice had a higher body weight than WT-g mice at the time of death (Figure 3.11B; $F(1, 55)= 30.321$, $p<0.001$), and males were heavier than females ($F(1, 55)=48.497$, $p<0.001$). This effect did not appear until week 14 (Figure 3.11A), suggesting that T5KO microbiota takes multiple weeks to have their effect on body weight (Vijay-Kumar et al., 2010). This was due to heavier adipose pads in the T5KO-g mice (Figure 3.11C, $F(1, 55)= 4.459$, $p=0.04$). T5KO-g mice had heavier (Figure 3.11D; $F(1, 55)=19.608$, $p<0.001$) and longer (Figure 3.11E; $F(1, 55)= 16.396$, $p<0.001$) colons than WT mice. Females had shorter colons than males ($F(1, 55)= 9.585$, $p=0.003$). T5KO-g mice had heavier ceca than WT mice (Figure 3.11F, $F(1, 55)=29.84$, $p<0.001$). T5KO-g mice also had mild splenomegaly (Figure 3.11G; $F(1, 55)= 12.278$, $p=0.001$), and females had heavier spleens than males ($F(1, 55)= 23.509$, $p<0.001$).

3.4.2.4 T5KO microbiota has a mild effect on anxiety-like, but not depressive-like or social, behavior in adult mice

Overall, T5KO-g mice spent less time in the center of the open field test than WT-g mice, suggesting that T5KO-g mice have a more anxious phenotype than WT-g mice. Male WT-g mice spent more time in the center of the arena than female WT-g mice, but this sex difference was reversed in the T5KO-g groups, resulting in a sex by treatment interaction (Figure 3.12A; $F(1, 55)=4.354$, $p=0.042$). This interaction was not due to differences between groups in distance traveled in the whole arena or the center

of the arena (Table 3.3; p>0.05). There was no difference between T5KO-g and WT-g mice in time spent in the open arms of the elevated zero maze (Figure 3.12B; p>0.05), nor time spent walking in the EZM (Table 3.3, p>0.05). T5KO-g and WT-g mice showed no differences in time spent struggling or hanging in the tail suspension test (Figure 3.12C; p>0.05). Furthermore, there were no differences in the time spent in the light zone of the light-dark box between treatment groups (Figure 3.12D; p>0.05). However, T5KO-g mice spent less time in a stretch-attend posture, in which mice stretch their midsections to explore the light zone while still remaining in the safety of the dark zone (Figure 3.12E; $F(1, 55)=4.552$, p=0.038), which may suggest that T5KO-g mice are less willing to risk exploring a novel area. There were no differences between zone entries between treatment groups (Table 3.3; p>0.05).

There was a trend towards a sex difference in the number of marbles buried during a 10-minute marble burying test, in which male T5KO-g mice buried more marbles than female T5KO-g mice (Figure 3.12F; $F(1, 55)=2.874$, p=0.096). However, this trend was not seen when the amount of digging behavior was quantified (data not shown; p>0.05). There was a sex difference in time spent grooming, where females from both treatment groups groomed themselves more than males (data not shown; $F(1, 55)=4.359$, p=0.042). There were no sex or treatment differences in time spent walking, rearing, or immobile (Table 3.3; p>0.05).

In the three-chamber test, there was a trend towards an interaction of sex and microbiota treatment on time spent in the chamber with the stimulus mouse (Figure 3.13A; $F(1, 53)= 3.827$, p-0.056), driven by a reversal in sex difference from WT-g to T5KO-g. Of interest, this did not translate into differences in time spent actively

investigating the mouse (Figure 3.13B; p>0.05). WT-g mice spent less time walking in the stimulus mouse chamber (Figure 3.13C; $F(1, 52)= 4.603$, p=0.037), but there were no differences in any other behaviors in this chamber. While there was no difference in time spent in the object chamber (p>0.05; data not shown), there was a trend towards an interaction between sex and treatment in time spent investigating the novel object (Figure 3.13D; $F(1, 53)= 5.325$, p=0.084. This interaction was driven by a significant increase in female WT mice, resulting in a main effect of sex ($F(1, 53)= 4.115$, p=0.048) and a main effect of treatment ($F(1, 53)= 4.22$, p=0.045). T5KO-g mice spent more time walking throughout each chamber than WT-g mice (Figure 3.13G; $F(1, 53)=30.25$, p<0.001).

3.4.2.5 Multivariate statistics reveal no pattern of behavior in microbiota treated mice

Discriminant analysis did not reveal separation of sex or genotype in the behavior of T5KO-g and WT-g mice (Figure 3.14 and Table 3.4, $\mathbf{\Lambda}=0.48$, $X^2(27)= 31.931$, p>0.05), further supporting the numerous non-significant behavioral results.

3.5 Discussion

We found that knockout of TLR5 results in an anxiogenic and depressive-like behavioral phenotype that was primarily due to changes in locomotion. Females seemed more susceptible to anxiety-like behavior, whereas males showed a slight elevation to depressive-like behavior. This behavioral phenotype was associated with greater AVP immunoreactivity. Characteristics of chronic intestinal inflammation and metabolic syndrome (Vijay-Kumar et al., 2010) were replicated in both sexes. While the physiological phenotype of T5KO was conferred to WT mice by microbiota transfer,

these mice did not show the T5KO behavioral phenotype. As expected, juvenile mice treated with T5KO microbiota did not show the behavioral or physiological characteristics of T5KO. These results suggest that there is another factor aside from microbiota that is responsible for the behavioral changes in T5KO mice.

One possible limitation of this study is the use of whole-body deletion of TLR5. TLR5 mRNA is expressed constitutively throughout the body, with the highest expression in the lungs and liver, and excluding the kidney (Sebastiani et al., 2000). There are low levels of TLR5 mRNA expression in the brain (Letiembre et al., 2007; Qiao et al., 2012), with expression in astrocytes and neurons in the cerebral cortex as well. Thus, it is entirely possible that the behavioral and neurochemical effects seen in the T5KO mice may be due to knockout in tissue other than the intestines. Although not directly tested here, it is likely that the results are from the loss of TLR5 in the intestine. Intestinal epithelial cell-specific knockout of TLR5 results in the same physiological phenotype as whole-body knockout (Chassaing et al., 2014), but the behavioral phenotype of these mice is unknown. Another limitation is the use of a different mouse strain, due to GF mouse availability, between experiments 1 and 2, which may contribute to the lack of behavioral differences after microbiota transplantation. However, C57Bl/6J and Swiss-Webster mice are shown to have similar anxiety-related traits (Crawley, 2008), suggesting that the behavioral differences between the strains used in this study are minor, and it is likely other factors are contributing to our behavioral results. Finally, it is difficult to interpret what changes to immunoreactivity indicate about the vasopressin and oxytocin systems. Greater immunoreactivity may indicate either increased production of the neuropeptide or less

release from the examined brain region. To truly identify how T5KO affects vasopressin and oxytocin, *in situ* hybridization could be used to measure vasopressin or oxytocin mRNA production, or microdialysis could measure vasopressin or oxytocin release.

T5KO mice display a behavioral profile reminiscent of mild sickness behavior. Sickness behavior is the behavioral complement to an infection and is defined as a motivational state that allows the organism to rest and recover from the infection (Dantzer et al., 2008). Thus, sickness behavior is characterized by increases in anxiety-like and depressive-like behavior, as well as aversions to movement, exploration and social interactions. Female T5KO mice showed more anxiogenic behavior in the OFT and EPM, but not L/D box, suggesting that T5KO may affect components of anxiety-like behavior differently between the sexes. The OFT has been described as better measure of passive coping behavior, while the L/D box and EPM are measures of active coping behavior (Bourin and Hascoët, 2003; Bourin et al., 2007; Nosek et al., 2008). It may be that the responses to different stressors may differ between T5KO males and females. When we examined the behavioral phenotype of T5KO mice using multiple discriminant analysis, we found that time spent floating in the FST, distance traveled in the OFT, time investigating the mouse in the TCT, and time spent in repetitive grooming were the factors that most differentiated the T5KO and WT mice. This is unsurprising, as examination of these behaviors individually showed significant genotypic differences (refer to Figures 3.2-3.4). Interestingly, the behaviors that differentiate the genotypes are components of locomotion, depressive-like and social behavior, suggesting that the phenotype is more reminiscent of sickness behavior than an anxiety-like behavior model.

T5KO mice show higher levels of inflammatory markers like Lcn-2, suggesting that this pro-inflammatory state may contribute to the anxiogenic phenotype. Lcn-2 modulates both peripheral and CNS responses to an infection, and Lnc-2 knockout experiments show that it is protective against exacerbated neuroinflammation and more severe sickness behavior (Ferreira, 2014; Kang et al., 2018). While we saw that females had a more severe anxiety-like phenotype than males, despite higher levels of Lcn-2, we did not investigate Lcn-2 levels in the brain, so we cannot say with certainty the role Lcn-2 is playing in the expression of this behavioral phenotype. It is also possible that serum Lcn-2 levels are correlated with systemic proinflammatory cytokine expression, which may account for the more severe phenotype in females. Elevated levels of pro-inflammatory cytokines are associated with increased anxiety-like and depressive-like behavior, and systemic injection of these cytokines can induce anxiety (Maes et al., 2012). T5KO results in colonic increase of the pro-inflammatory cytokines IL-1B and TNF-a, suggesting that the peripheral inflammation may contribute to the elevated anxiety-like behavior in T5KO mice (Vijay-Kumar et al., 2007). Furthermore, peripheral injection of LPS activates the PVN, and therefore the HPA axis, through an IL-1β dependent mechanism (Quan et al., 2003). It is possible that these cytokines are signaling to the brain through afferent nerves in the colon, exacting the neural and behavioral changes summarized here. Taken together, the increased inflammation seen in T5KO mice results in mild sickness behavior, with some behaviors differentially affected by sex, and future experiments will need to probe into the exact immune factors regulating this behavioral phenotype.

Converse to canonical sickness behavior, T5KO mice were more investigatory in the three-chamber test than WT mice, especially when WT mice were the stimulus animals. T5KO mice actively investigated the WT stimulus mice in the first three-chamber sociability cohort more than WT mice investigated the WT stimulus, suggesting that T5KO mice had more motivation for social interaction than the WT mice. However, it is possible that the T5KO mice were responding to a different series of odor cues from the WT mice than they are normally exposed to. It is well-established that mice and other animals use odor cues in a variety of social situations, including kin recognition and mate selection (Bienenstock et al., 2018). In addition, gut microbiota can produce many odorants (Ezenwa and Williams, 2014), so it is likely that the gut microbiota contributes to the differences in social behavior we observed. In fact, rats raised in a germ-free environment lost their odors of individuality (Singh et al., 1990), and experimental rats could not distinguish between the odors of two germ-free MHC-congenic stimulus rats (Schellinck et al., 1995). Furthermore, mice treated with LPS alters urine odor such that mice could distinguish between LPS- and control-treated urine in a Y maze (Kimball et al., 2014). This evidence suggests that either the changes in gut microbiota of the T5KO mice and/or the immune system activation could have altered their body odor, resulting in increased investigation of the novel-scented WT mice. When given the choice between a WT or T5KO mouse, both sexes of T5KO mouse showed a preference for investigating the WT mouse. This pattern goes the other way as well, where male WT mice greatly preferred to investigate the T5KO mouse, suggesting both WT and T5KO have a preference for odor novelty in a controlled environment.

We found that AVP immunoreactivity is greater in the SCN and PVN and their projection sites, in response to T5KO. These regions control circadian rhythms and motivated behaviors, respectively, and both have been shown to contribute to sickness behavior through regulation of anxiety-like and depressive-like behaviors (Dantzer, 2006; Andries Kalsbeek et al., 2010; Landgraf et al., 2016). The hypothalamus and thalamus receives input from the gut by way of the vagus nerve and the nucleus of the solitary tract, so it is likely that inflammatory signals in T5KO mice affect OXT and AVP producing neurons in the hypothalamus, which could then project to other areas of the brain to enact behavioral changes (Goehler et al., 2005). Specifically, greater AVP immunoreactivity in the SCN coupled with decreases in AVP mRNA production may underlie the lethargy involved in depressive-like behaviors, and depressed patients have higher numbers of AVP-ir positive neurons in the SCN (Dai et al., 1998; Zhou et al., 2001). This suggests that the greater AVP immunoreactivity in the SCN, as well as the projection sites the DMH, PVT, and SPZ, may have disrupted the circadian rhythms of T5KO, contributing to increased lethargy and depressive-like behavior. AVP signaling from the SCN can disrupt the HPA axis as well through GABAergic interneurons in the DMH and SPZ, which also may contribute to the increased anxiety-like behavior (Kalsbeek et al., 2010). More thorough examination of HPA activation is prudent to understand the interplay between T5KO and anxiety-like and stress behaviors.

The increases we see in AVP immunoreactivity in the PVN and its projection sites may be contributing to the anxiogenic behavioral phenotype in T5KO mice. AVP produced in the PVN is projected to a number of brain regions, including the lateral

septum and central amygdala, as well as is released into the median eminence to stimulate adrenocorticotropin from the anterior pituitary (Csikota et al., 2016; Hernández et al., 2016), indicating that AVP from the PVN can affect anxiety-like behavior through its effects in other brain regions and through HPA activation. For example, AVP infusion into the central amygdala in rats (Hernández et al., 2016), and overexpressing the Avpr1a gene in the lateral septum in mice (Bielsky et al., 2005), increases anxiety-like behavior. Mouse models of anxiety tend to have increased HPA axis activity, which is associated with increased AVP, and perturbing the stress response results in anxiety-like behavior (Landgraf, Wigger, Holsboer, & Neumann, 1999; Niraula, Witcher, Sheridan, & Godbout, 2019), but this is not always the case (Neufeld et al., 2011; Sudo et al., 2004). More investigation is needed to determine how T5KO affects AVP and the HPA axis, especially to delineate whether the greater immunoreactivity is due to increased production of AVP mRNA or less release from the PVN.

We found that OXT is not very affected by inflammation in T5KO mice. Ample evidence suggests that OXT both anxiolytic and anti-inflammatory in the brain (Neumann and Landgraf, 2012; Yuan et al., 2016a; Wang et al., 2018), so it is not surprising that OXT was only mildly affected in our experiments. We did see elevated OXT immunoreactivity in the BNSTmv, which is important for the regulation of social behavior (Lebow and Chen, 2016), indicating that the increased sociability in T5KO mice may be due to this increase in OXT. As mentioned previously, the greater OXT-ir may be either increased mRNA production or decreased neuropeptide release. Thus, more investigation is needed to identify what effect T5KO has on oxytocin.

Our T5KO mice replicated the physiological phenotype previously reported (Chassaing et al., 2014; Vijay-Kumar et al., 2010). This is true for adult WT mice colonized with T5KO microbiota as well, albeit to a less severe degree, but not for juvenile T5KO-g mice. This is not surprising, as T5KO requires multiple weeks for metabolic syndrome to develop (Carvalho et al., 2012; Vijay-Kumar et al., 2010). While T5KO microbiota is sufficient to promote intestinal inflammation and characteristics of metabolic syndrome in the adult offspring of microbiota-transplanted mice, this was not the case for the behavioral phenotype, suggesting that the behavior of T5KO mice is modulated by more than just the microbiota. It is unclear what other factors may underlie this microbiota-independent pathway, as we only measured behavioral expression in these animals. It is possible that the intestinal inflammation did not extend past the gut, for example to the brain, because the T5KO-g mice did have functioning TLR5 receptors. Even if the T5KO-g mice had an increased bacterial burden, thinner mucus layer and more bacteria invading the gut epithelium, the presence of TLR5 should catch any invading bacteria before a large-scale immune response is mounted. It is also possible that more time was needed for the microbiota transplant to fully affect behavior, which could be achieved by testing the mice at a later age (20 weeks instead of 15), colonizing the parents at an earlier age than weaning, or colonizing the experimental mice at birth. It is also possible that our procedure of colonizing the GF mice at weaning resulted in microbiota shift to a more similar microbiota between WT-g and T5KO-g mice. A recent experiment by Fulde and colleagues found that GF mice colonized with T5KO microbiota as neonates had microbiota compositions that grew more similar to WT microbiota over 28 days than

T5KO microbiota given to adults (Fulde et al., 2018). This suggests that we may need to administer T5KO microbiota to the parents in adulthood to maintain separation between T5KO and WT mice. Future studies warrant investigation of systemic and neural cytokines as well as neuropeptide expression to better pinpoint the deviation from the T5KO phenotype.

Our results suggest that the physiological changes in T5KO may induce mild sickness behavior through the elevation of hypothalamic AVP immunoreactivity in a gut microbiota-independent mechanism. While TLR5 deficiency in humans does not result in the same chronic inflammation that it causes in mice (Gewirtz et al., 2006), T5KO mice are a novel model for investigating the microbiota-gut-brain communication pathways that contribute to the association between inflammation and behavior. Anxiety and depression are strongly associated with inflammation and metabolic syndrome in humans, so identifying the mechanisms underlying this relationship is imperative for developing novel therapeutics.

3.6 Figures

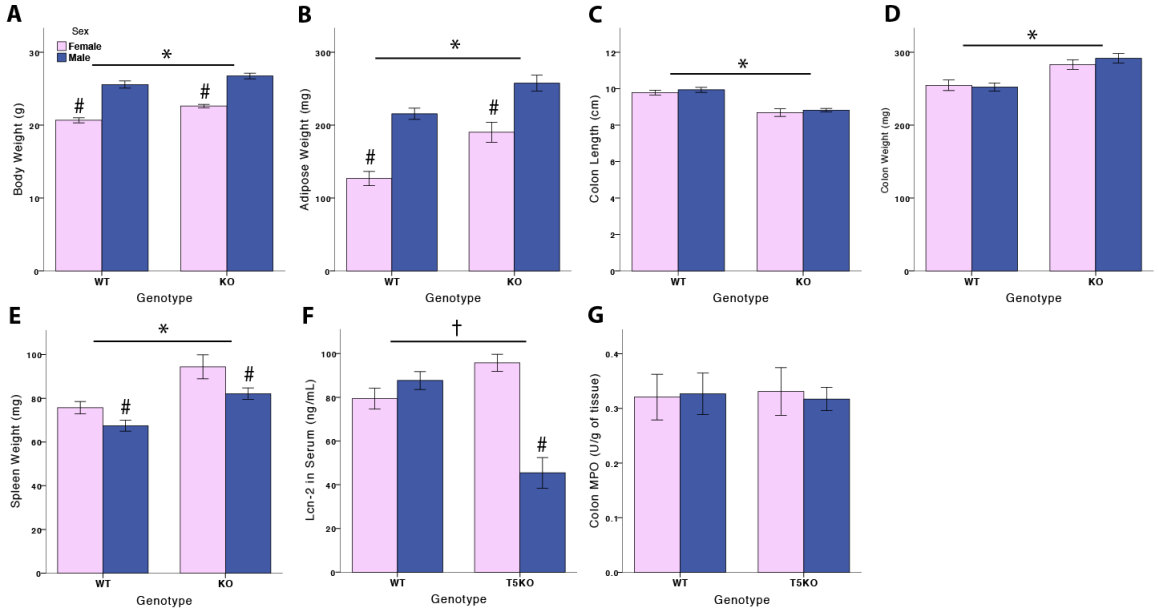

Figure 3.1. T5KO mice replicate metabolic syndrome and low-grade inflammatory phenotype.

T5KO mice show morphological phenotypes of metabolic syndrome **(A-B)** and intestinal inflammation **(C-E). (A)** Body weight, **(B)** gonadal fat pad mass, **(C)** colon length, **(D)** colon weight, and **(E)** spleen weight were recorded. Markers of intestinal inflammation **(F)** Lcn-2 from blood serum and **(G)** colonic MPO tissue show T5KO mice have low-grade, but not colitic, inflammation. * represents a significant main effect of treatment ($p<0.05$). # represents a significant main effect of sex ($p<0.05$). ┼ represents a significant treatment by sex interaction ($p<0.05$). Data presented as +/- SEM (n= 16-22).

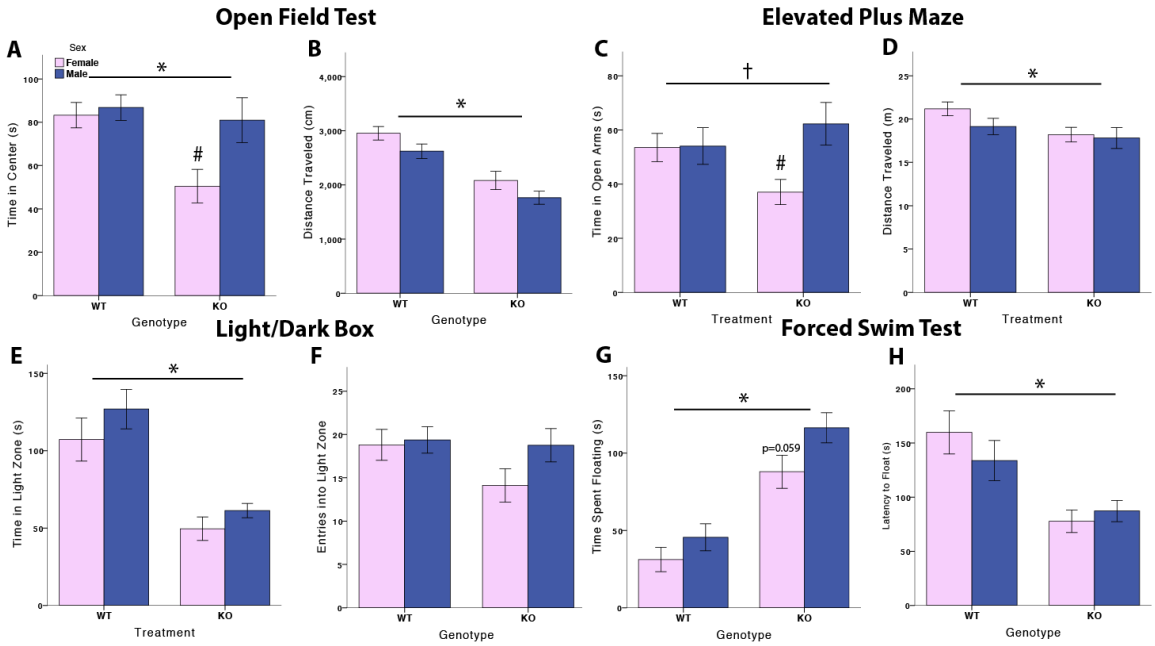

Figure 3.2. T5KO mice have an anxious and depressive phenotype.

Behavior of T5KO and WT mice were analyzed in the open field test **(A-B)**, elevated plus maze **(C-D)**, light/dark box **(E-F)** and forced swim test **(G-H)**. **(A)** Time spent in the center of the arena. **(B)** Distance traveled in the entire arena. **(C)** Time spent in the open arms of the EPM. **(D)** Distance traveled in the apparatus. **(E)** Time spent in the light chamber. **(F)** Number of entries into the light chamber. **(G)** Time spent floating in the forced swim test. **(H)** Latency to begin floating. * represents a significant main effect of treatment ($p<0.05$). # represents a significant main effect of sex ($p<0.05$). ┼ represents a significant treatment by sex interaction ($p<0.05$). Trends toward significance are represented with the p-value. Data presented as +/- SEM (n= 16-22).

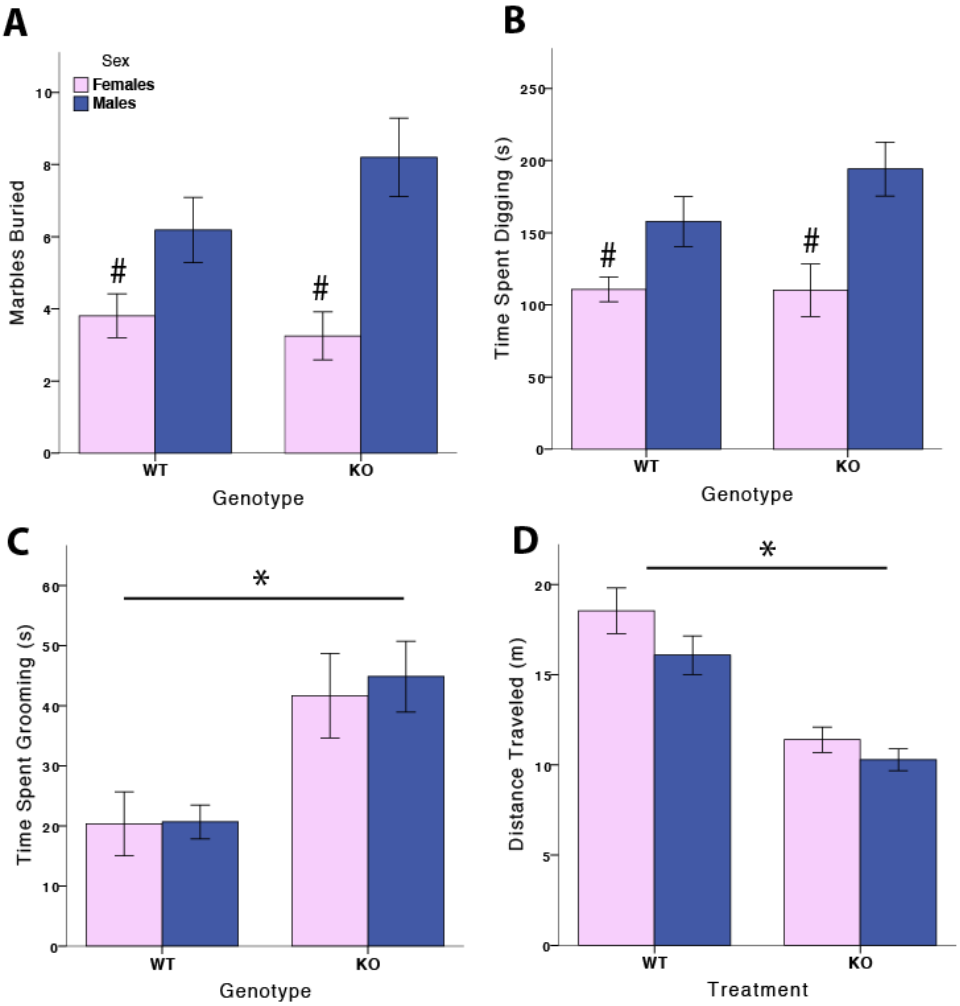

Figure 3.3. T5KO mice show increased repetitive grooming in the marble burying test.

(A) Number of marbles buried. **(B)** Time spent digging in the bedding of the arena. **(C)** Time spent grooming. **(D)** Distance traveled in the arena. * represents a significant main effect of treatment ($p<0.05$). # represents a significant main effect of sex ($p<0.05$). Data presented as +/- SEM (n= 16-22).

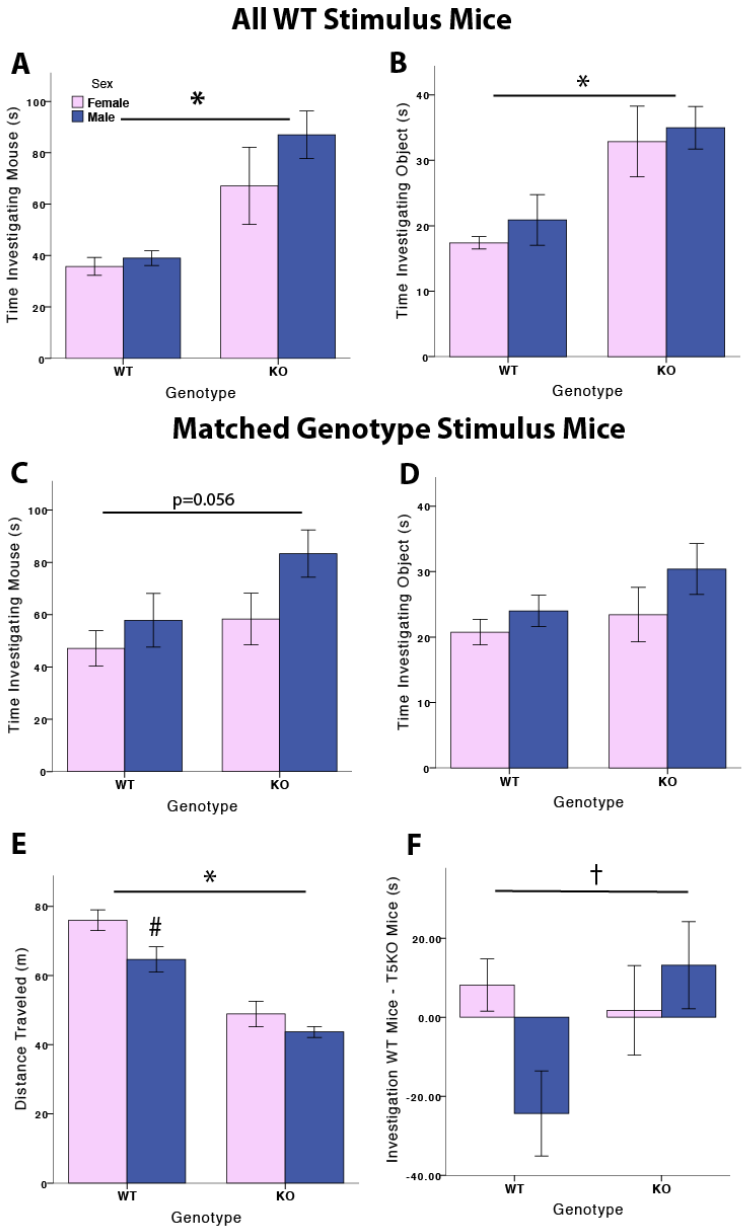

Figure 3.4. T5KO mice are more social than WT mice when WT mice are used as a stimulus.

(A-B) T5KO and WT mice were given the choice between a WT mouse and a novel object (n=8-13). Time actively investigating the mouse **(A)** or object **(B)** were analyzed. **(C-D)** A separate cohort (n=7-10) of T5KO and WT mice were given the choice between a matched genotype mouse **(C)** or an object **(D)**. **(E)** Combined distance traveled data

from cohorts in A-D (n=16-22). **(F)** A separate cohort of mice were given the choice between a T5KO or WT mouse (n= 9-10). Preference score was determined by subtracting time investigating the T5KO mouse from the WT mouse. Positive score indicates preference for the WT mouse. * represents a significant main effect of treatment ($p<0.05$). # represents a significant main effect of sex ($p<0.05$). ┼ represents a significant treatment by sex interaction ($p<0.05$). Trends toward significance are represented with the p-value.

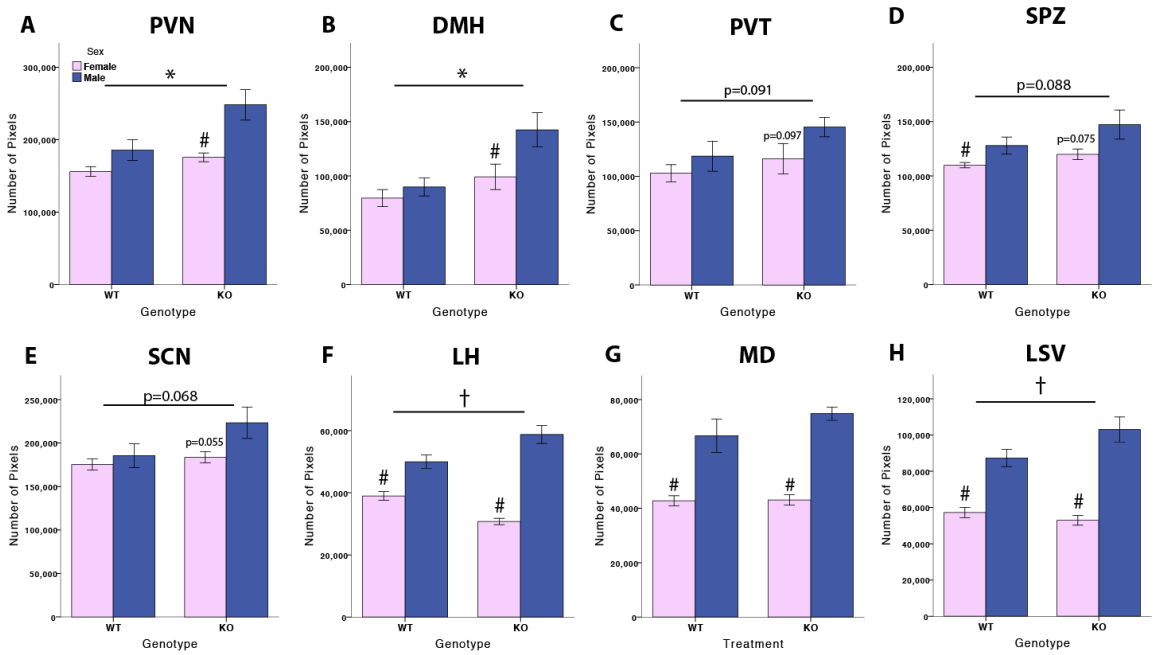

Figure 3.5. T5KO increased AVP-ir in PVN and SCN projection sites.

AVP-ir was determined by gray-level thresholding in the **(A)** PVN, **(B)** DMH, **(C)** PVT, **(D)** SPZ, **(E)** SCN, **(F)** LHb, **(G)** MD, and **(H)** LSV. * represents a significant main effect of treatment ($p<0.05$). # represents a significant main effect of sex ($p<0.05$). † represents a significant treatment by sex interaction ($p<0.05$). Trends toward significance are represented with the p-value. Data presented as +/- SEM (n= 8-9).

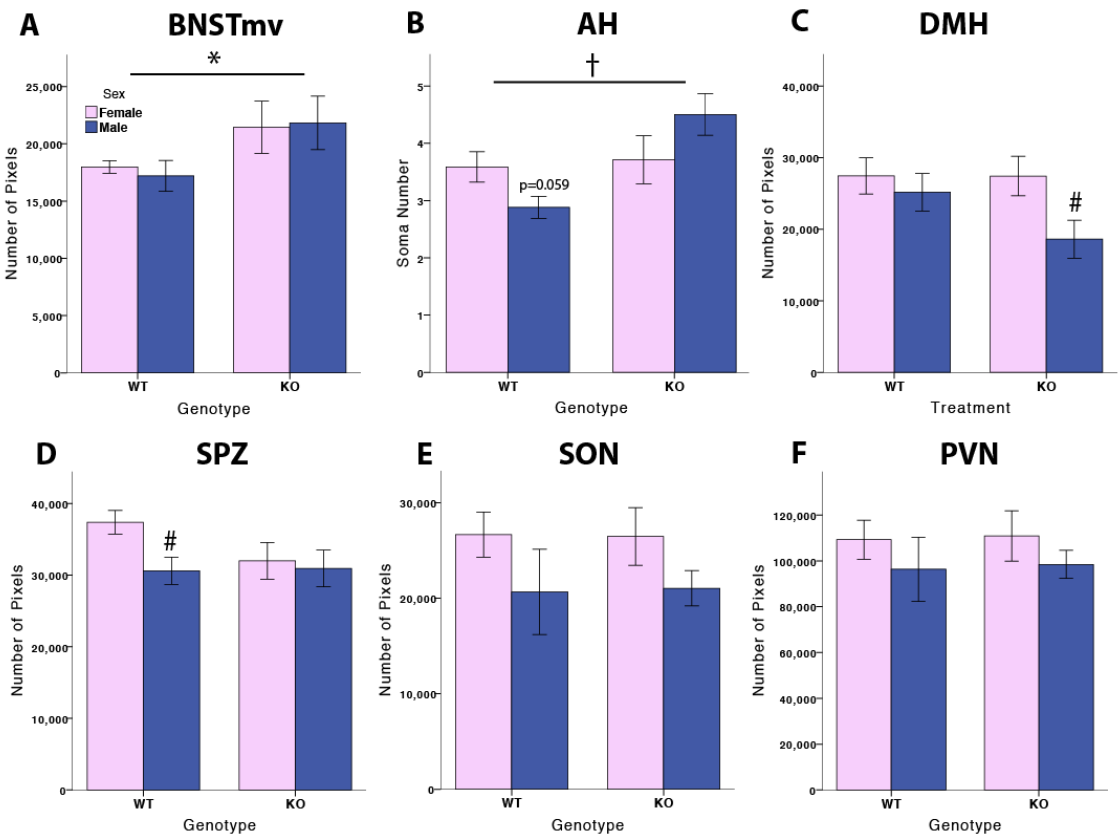

Figure 3.6. T5KO has little effect on OXT-ir.

OXT-ir **(A, C-F)** or OXT-positive cell counts **(B)** were determined in the **(A)** BNSTmv, **(B)** AH, **(C)** DMH, **(D)** SPZ, **(E)** SON, and **(F)** PVN. # represents a significant main effect of sex ($p<0.05$). ┼ represents a significant treatment by sex interaction ($p<0.05$). Data presented as +/- SEM (n= 9-11).

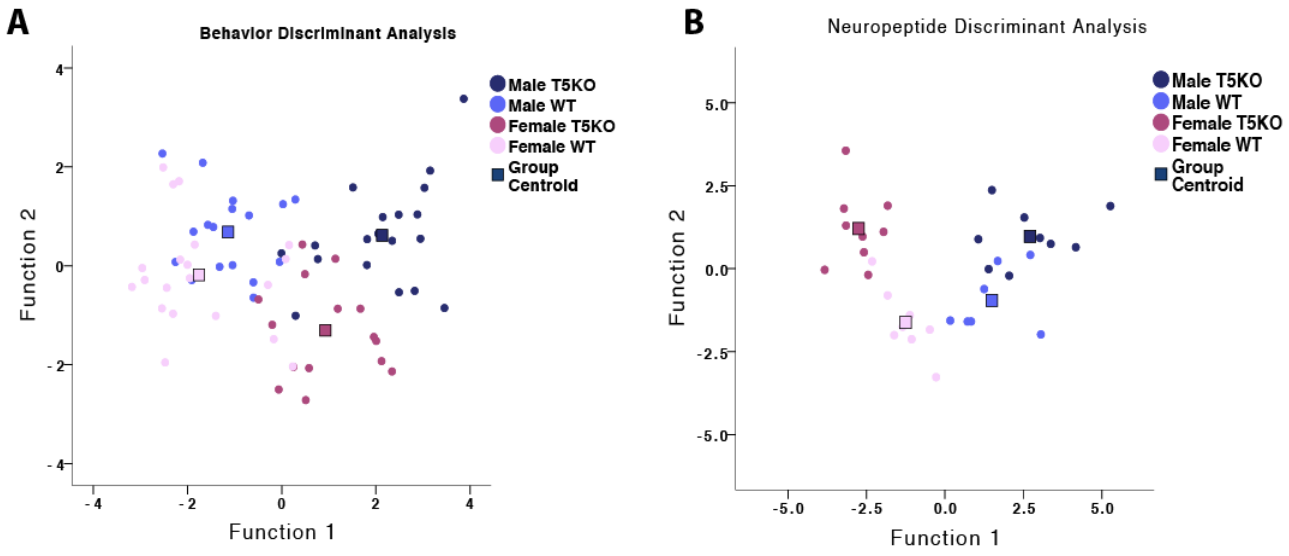

Figure 3.7. Multivariate test statistics reveal a separation of sex and genotype in behavioral and neuropeptide expression in T5KO and WT mice.

The canonical discrimination function plot revealed a significant separation of groups by sex and genotype along 3 different functions for **(A)** anxiety-like, depressive-like, and social behaviors and **(B)** AVP and OXT protein expression. Individual contributions of each measured outcome are summarized in Table 1 for behavioral measures and Table 2 for neuropeptides.

Table 3.1. Structure matrix for discriminant analysis of behavior.

Measured Outcomes	Function		
	1	2	3
Time Floating in FST (s)	**.529***	.005	-.031
Distance Traveled in the OFT (cm)	**.510***	-.002	.399
Time Investigating Mouse in TCT (s)	**.415***	.123	.198
Time Grooming in Marble Burying Test (s)	**.304***	-.183	.097
Number of Marbles Buried	.202	**.619***	-.102
Time in Light Zone of L/D Box (s)	-.383	**.498***	-.255
Time in Center of OFT (s)	-.115	**.479***	.422
Open Arm Time in EPM (s)	.020	.338	**.558***

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

Table 3.2. Structure matrix for discriminant analysis of neuropeptide expression.

Measured Outcomes	Function		
	1	2	3
LHb AVP	**.805***	.047	.264
MD AVP	**.565***	.224	-.426
DMH AVP	.214	**.396***	.351
PVN AVP	.319	**.392***	.198
PVT AVP	.163	**.281***	.081
DMH OXT	-.192	**-.193***	-.083
SON OXT	-.149	.046	**.410***
SPZ OXT	-.068	-.207	**.370***
BNST OXT	-.002	.305	**.331***
SCN AVP	.174	.209	**.325***
PVN OXT	-.097	.118	**.244***

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

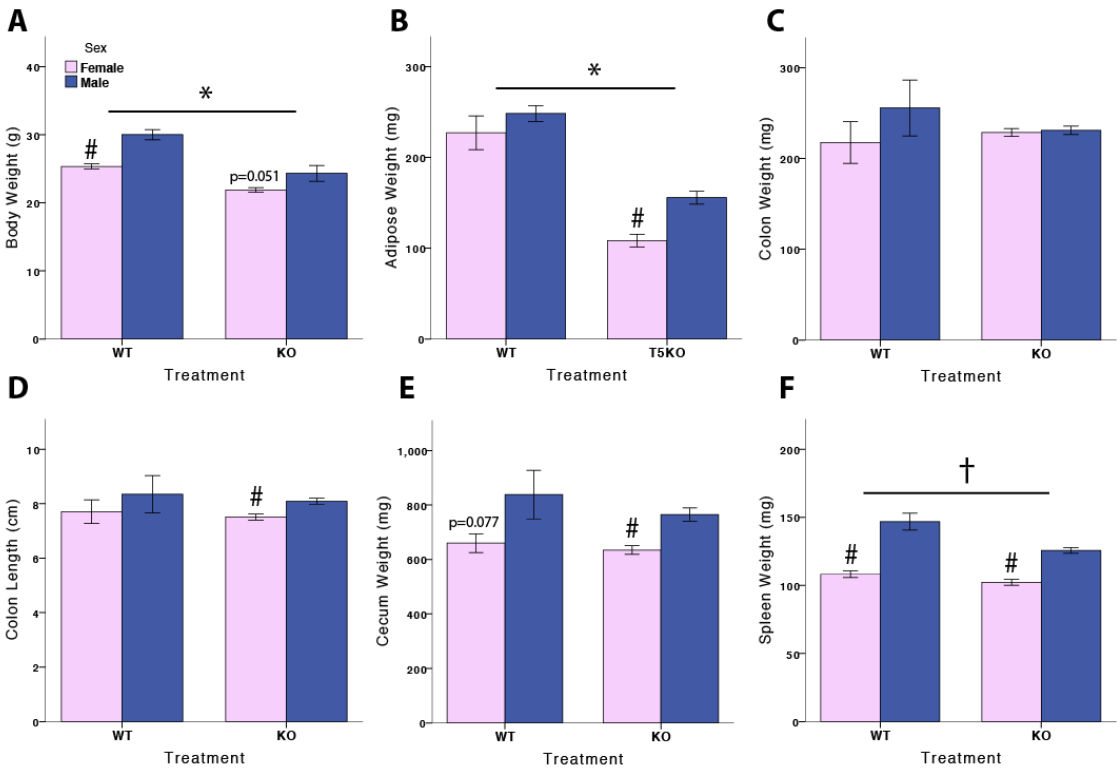

Figure 3.8. T5KO-g is not sufficient to induce morphological T5KO phenotype in juvenile (P29) mice.

Morphological aspects of **(A-B)** metabolic syndrome and **(C-F)** intestinal inflammation were measured. **(A)** Body weight, **(B)** adipose weight, **(C)** colon weight, **(D)** colon length, **(E)** cecum weight, and **(F)** spleen weight were recorded at time of sacrifice. * represents a significant main effect of treatment ($p<0.05$). # represents a significant main effect of sex ($p<0.05$). ┼ represents a significant treatment by sex interaction ($p<0.05$). Trends toward significance are represented with the p-value. Data are represented as means +/- SEM (n=5-23).

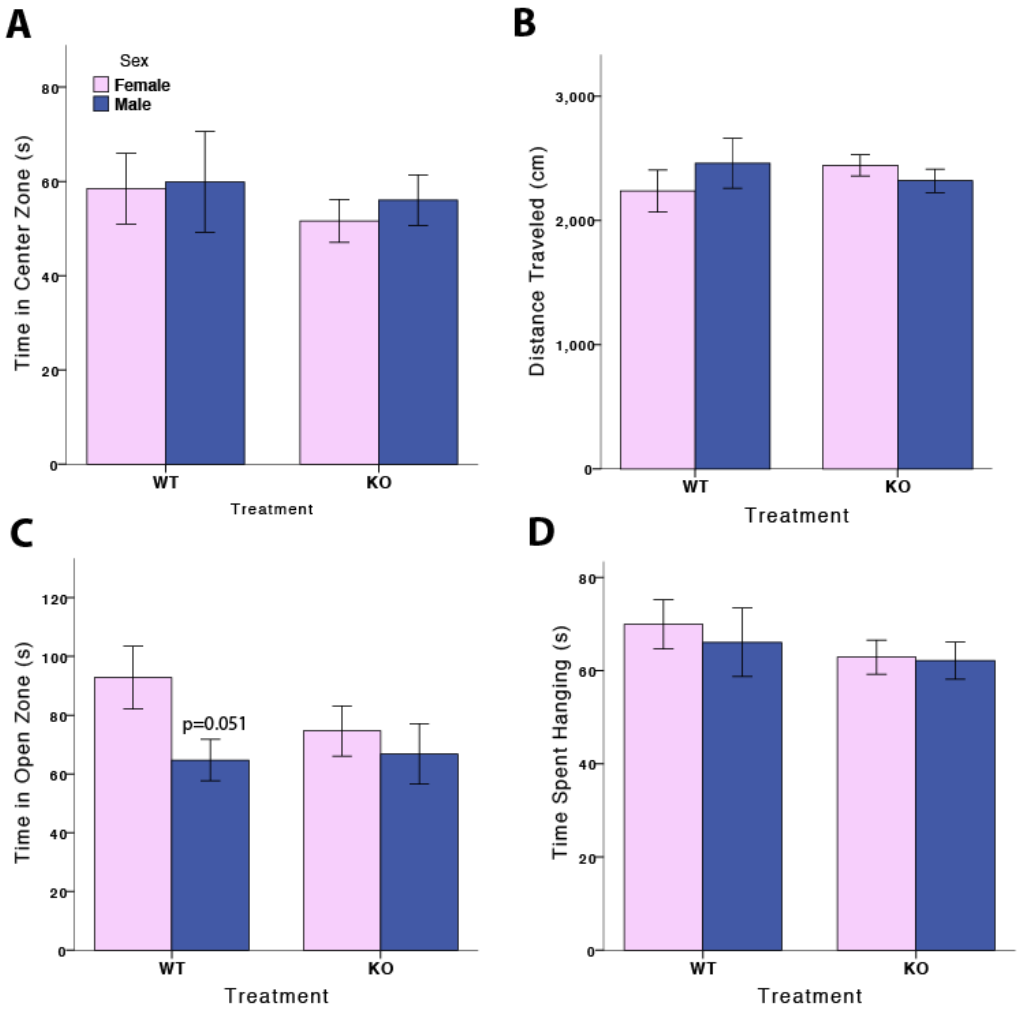

Figure 3.9. T5KO-g has no effect on anxiety- and depressive-like behavior in weanling-aged mice.

(A) Time in the center zone and **(B)** distance traveled in the OFT. **(C)** Time spent in open zone of the elevated zero maze. **(D)** Time spent hanging in the tail suspension test. Data are represented as means +/- SEM (n=13-39).

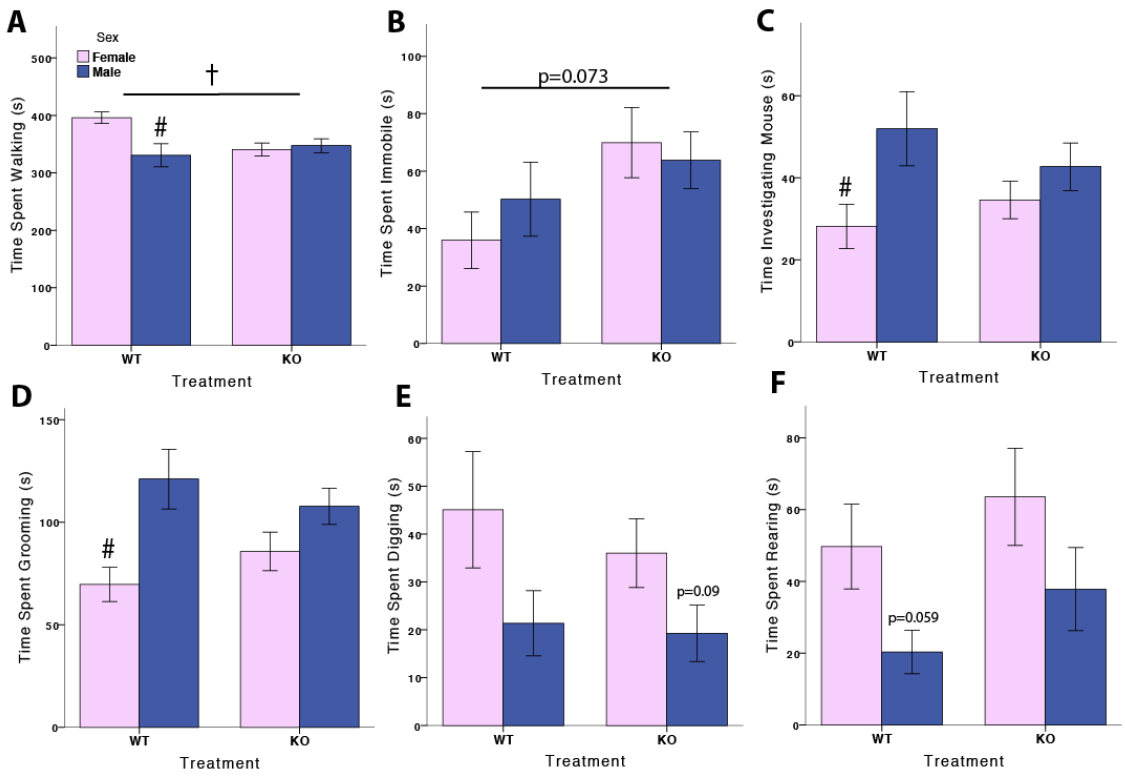

Figure 3.10. T5KO-g had no effect on weanling-aged social behavior.

(A) Time spent moving in the arena, **(B)** time spent immobile, **(C)** time in active investigation of the other mouse**, (D)** time spent grooming, **(E)** time spent digging in the bedding, and **(F)** time spent rearing against the walls of the arena were recorded during a 10-minute social interaction test with a littermate. # represents a significant main effect of sex ($p<0.05$). ┼ represents a significant treatment by sex interaction ($p<0.05$). Trends toward significance are represented with the p-value. Data are represented as means +/- SEM (n=13-39).

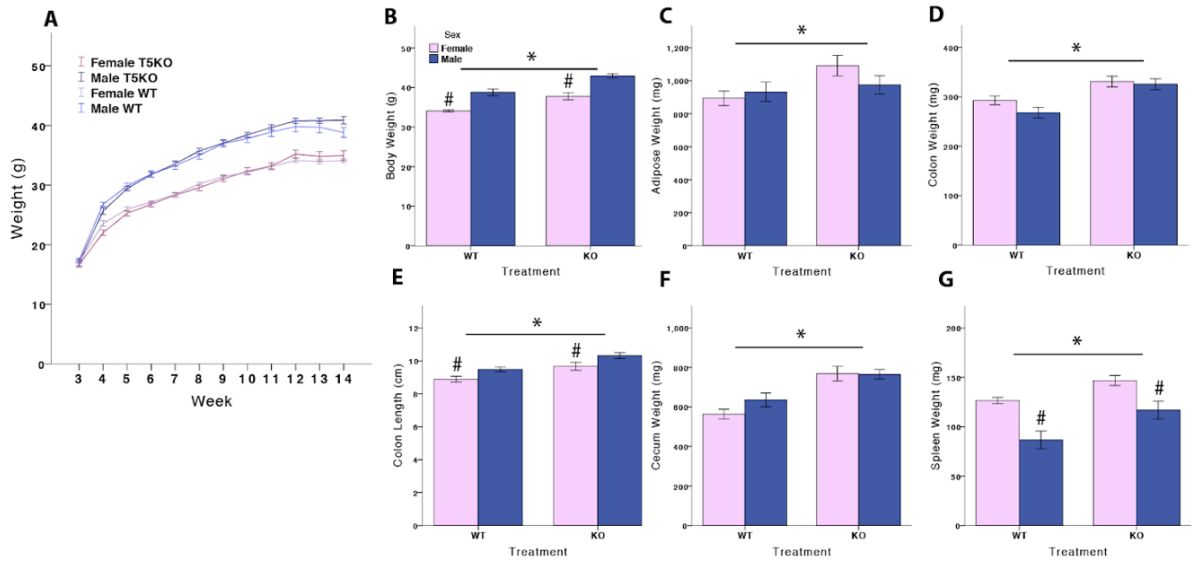

Figure 3.11. T5KO microbiota is sufficient to produce the morphological phenotype of T5KO in adult mice.

Morphological aspects of **(A-C)** metabolic syndrome and **(D-H)** intestinal inflammation were measured. **(A)** Body weight over time, beginning at weaning (3 weeks of age) to adulthood (14 weeks of age), **(B)** Body weight at time of sacrifice, **(C)** gonadal fat pad weight, **(D)** colon weight, **(E)** colon length, **(F)** cecum weight, **(G)** spleen weight, and **(H)** liver weight. * represents a significant main effect of treatment ($p<0.05$). # represents a significant main effect of sex ($p<0.05$). Trends toward significance are represented with the p-value. Data are represented as means +/- SEM (n=11-16).

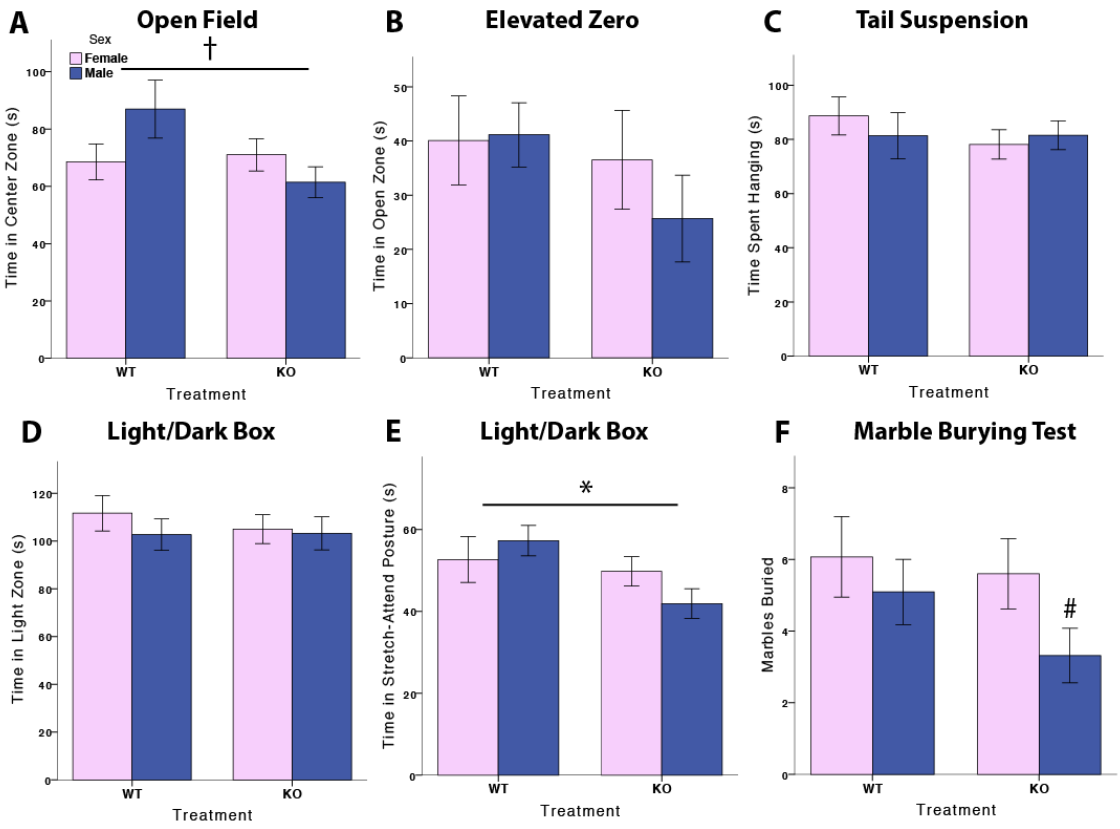

Figure 3.12. T5KO-g has a mild effect on adult anxiety-like and depressive-like behavior.

(A) Time in the center zone of the open field test, **(B)** time in the open zone of the elevated zero maze, **(C)** time spent hanging in the tail suspension test, **(D)** time in the light zone of the light/dark box, and **(E)** time spent in the stretch-attend posture in the L/D box. * represents a significant main effect of treatment ($p<0.05$). # represents a significant main effect of sex ($p<0.05$). ┼ represents a significant treatment by sex interaction ($p<0.05$). Data are represented as means +/- SEM (n=11-16).

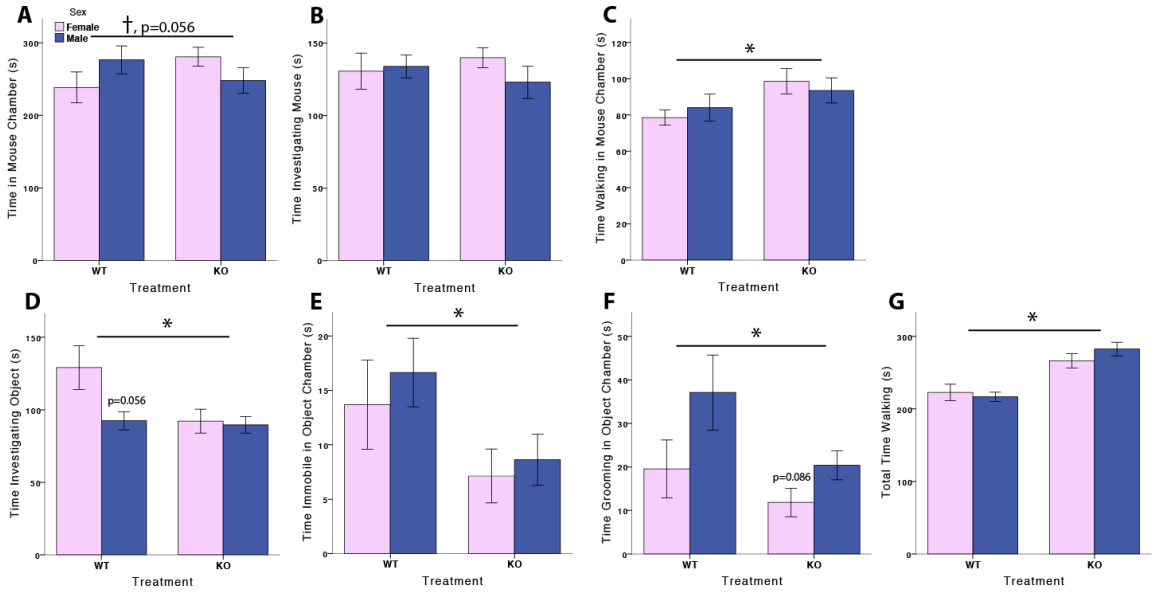

Figure 3.13. T5KO-g had little effect on adult social behavior in the three-chamber apparatus.

(A) Time spent in the mouse chamber, **(B)** time investigating the mouse, **(C)** time walking in the mouse chamber, **(D)** time investigating the object, **(E)** time spent immobile in the object chamber, **(F)** time spent grooming in the object chamber, and **(G)** total time spent walking in all three chambers. * represents a significant main effect of treatment ($p<0.05$). # represents a significant main effect of sex ($p<0.05$). ┼ represents a significant treatment by sex interaction ($p<0.05$). Data are represented as means +/- SEM (n=11-16).

Table 3.3 Behavioral Data for Microbiota-treated Mice

Behavior	Treatment Group			
	Male T5KO-g	**Female T5KO-g**	**Male WT-g**	**Female WT-g**
Distance Traveled in OFT (cm)	2,533+/- 141	2526+/- 176	2400+/- 144	2735+/- 188
Time Walking in EZM (s)	89.6 +/- 8.2	74.3 +/- 7.1	93.7 +/- 5.4	89.9 +/- 7.1
Light Entries in L/D Box	9.1 +/- 1.4	9.3 +/- 1.2	6.3 +/- .9	9.0 +/- 1.4
Time Walking in Marbles (s)	211.6 +/- 24.3	172.4 +/- 9.3	169.9 +/- 11.8	167.1 +/- 14.6
Time Rearing in Marbles (s)	47.5 +/- 7.1	49.5 +/- 4.5	37.3 +/- 6.1	52.2 +/- 8.4
Time Immobile in Marbles (s)	167.1 +/- 30.2	154.1 +/- 13.5	176.5 +/- 25.7	153.7 +/- 24.4
Time Digging in Marbles (s)	162.8 +/- 22.2	176.2 +/- 20.2	183.5 +/- 21.7	175.7 +/- 20.1

Measures of locomotion and other behaviors in anxiety-like behavioral assays. Data are represented as the mean +/- SEM. There were no significant differences between groups in any measures listed.

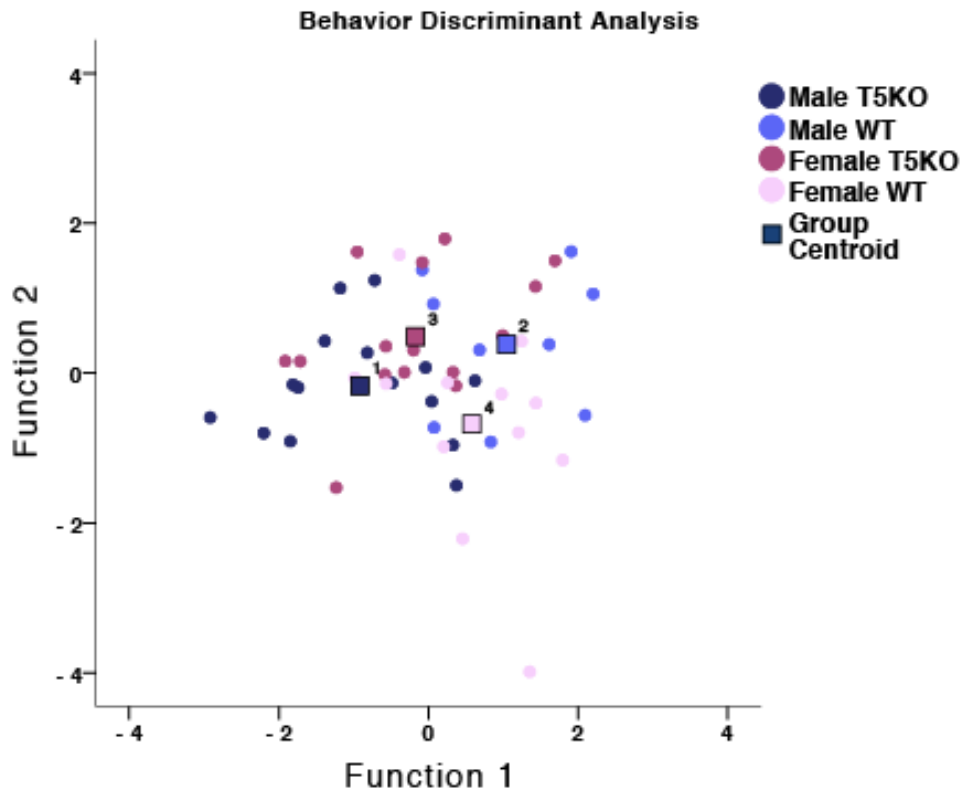

Figure 3.14. Discriminant analysis does not reveal a separation along sex or microbiota treatment for behavioral characteristics of T5KO-g or WT-g mice.

The canonical discrimination function plot revealed a significant separation of groups by sex and genotype along 3 different functions for anxiety-like, depressive-like, and social behaviors. Individual contributions of each measured outcome are summarized in Table 3.

Table 3.4. Structure matrix for discriminant analysis of behavior in microbiota treated mice.

Measured Outcomes	Function		
	1	2	3
Total Time Walking in TCT (s)	**-.574***	.024	-.296
Stretch Attend Posture in L/D Box (s)	**-508***	-.178	.271
Time in Center of OFT (s)	**.332***	.302	-.199
Time Walking in Mouse Chamber (s)	**-.151***	-.013	-.139
Time Investigating Mouse in TCT (s)	.059	**.683***	.264
Time in Light Chamber of L/D Box (s)	.022	**-.210***	.101
Time Spent Hanging in TST (s)	.079	**-.189***	-.067
Time Investigating Object in TCT (s)	-.019	-.266	**.615***

Open Zone Time of EZM (s)	.261	-.036	**.349***

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

4 DISCUSSION

The projects described in this dissertation serve to highlight the importance of considering vasopressin and oxytocin as targets of microbiota influence in the microbiota-gut-brain-behavior axis. Alterations in gut microbiota composition dysregulate the vasopressin and oxytocin systems, most likely through an immune signaling pathway, to result in disordered anxiety-like, depressive-like and social behaviors. Within this discussion I will outline the importance of vasopressin and oxytocin in this pathway, propose a possible mechanism by which microbiota affects these neuropeptides in the regulation of behavior, and place these results in the larger context of human health. These studies highlight the need for more investigation into the mechanisms underlying the microbiota-gut-brain-behavior axis in order to harness this axis in developing therapeutics for both gastrointestinal and psychiatric disorders.

4.1 Vasopressin and Oxytocin in the Microbiota-Gut-Brain-Behavior Axis

In Chapter 2, we found that recolonization with microbiota increased vasopressin expression, suggesting that something about colonization with microbiota resulted in greater vasopressin immunoreactivity. It is possible that the GF mice we recolonized were exposed to pathogenic bacteria, which would elicit an immune response. GF mice have a blunted immune response to pathogens, and recolonization with microbiota does not fully rescue splenic levels of macrophages (Khosravi et al., 2014; Pickard et al., 2017), so an exposure to a pathogen may result in a more severe inflammatory response than in a CC mouse. This could have downstream effects on vasopressin in the brain, as cytokines tend to have a stimulatory effect on vasopressin production (Palin et al., 2009). Colonization with microbiota occurred by oral gavage at weaning,

each of which are stressful events to the mouse (Walker et al., 2012; Farshim et al., 2016; Richter et al., 2016; Dong et al., 2017). Stressful events in early life, like weaning or oral gavage, can increase vasopressin mRNA production in the hypothalamus (Veenema et al., 2007). The combination of these events suggest that the colonization of GF mice may increase vasopressin immunoreactivity through immune- and stress-mediated mechanisms. In Chapter 3, we found that intestinal inflammation increased vasopressin immunoreactivity, again pointing to gut-derived inflammation stimulating vasopressin production in the brain. However, we cannot fully conclude this is the case, because we do not know whether the increase in immunoreactivity is due to elevated vasopressin production or rather reductions in vasopressin release. We could investigate the effects on the vasopressin system further by either measuring vasopressin mRNA production through *in situ* hybridization, or vasopressin release through microdialysis.

In Chapter 3, we found that vasopressin expression was associated with an anxiogenic phenotype and reduction in locomotion in the T5KO mice. Vasopressin is implicated in the generation of anxiety states in rodents, especially its actions in the lateral septum (Liebsch et al., 1996). Injections of vasopressin into the LS is anxiogenic, and blockade of V1aR through antagonists or genetic knockouts is anxiolytic (Landgraf et al., 1995; Liebsch et al., 1996; Beiderbeck et al., 2007). We found that T5KO males had increased vasopressin in the LS, but there was no effect in females. This is surprising, because T5KO females had a stronger anxiogenic phenotype. This may suggest that vasopressin in the LS is less critical for the expression of anxiety-like behaviors in this model. Vasopressin also modulates

voluntary locomotion, where systemic and hypothalamic vasopressin injections reduced voluntary wheel running in golden hamsters (Cormier et al., 2015), suggesting the reduction in movement we saw in the T5KO mice may be due to the actions of vasopressin. However, more investigation into the base level of locomotion is important to determine if T5KO mice are moving less overall, or just in a novel environment.

The main disruptions to the vasopressin system in both microbiota manipulations were found in the SCN and its projection sites. Vasopressin in the SCN is involved in maintaining circadian rhythmicity, responding to immune signals, and regulating the HPA axis (Kalsbeek et al., 2010). Dysregulation of circadian rhythms can have deleterious effects on mood and anxiety, so it is possible that the behavioral changes we saw were due to a disruption of circadian rhythms (Kim et al., 2017). There is a bidirectional interaction between circadian rhythms and microbiota. Microbiota have their own diurnal rhythms that can affect the host's rhythms, and disruption to the host's circadian patterns can affect microbiota composition (Voigt et al., 2014; Liang et al., 2015; Rosselot et al., 2016; Thaiss et al., 2016), which suggests that our microbiota alterations may have affected the rhythmicity of our mice. In fact, GF mice show altered circadian rhythms in gene expression and metabolism (Wang et al., 2017; Weger et al., 2019), which may contribute to the differences in behavior that GF mice possess. It is also possible that disruptions to the circadian rhythms may have resulted in behaviorally testing the different animal groups in different parts of their subjective photoperiods. The part of the photoperiod that behavioral testing occurs in can have numerous effects on behavioral expression (Huynh et al., 2011; Labots et al., 2016; Anyan et al., 2017). However, we do not know whether circadian rhythms are affected in the T5KO mice.

More experiments identifying the roles that microbiota play in the disruption of vasopressin in the SCN are needed.

Inflammatory signals had minor effects on vasopressin from the BNST projection sites. Inflammation affected vasopressin in the LHb differently between the sexes. Lateral habenula hyperactivity is associated with depressive-like behaviors and aversion in general (Yang et al., 2018), so increased vasopressin in this region may contribute to a depressive-like phenotype. In Chapter 2, recolonization eradicated the sex difference in vasopressin in BNST projection sites, and in Chapter 3, T5KO reduced AVP immunoreactivity in females but elevated it in males, which may have contributed to the increased depressive-like phenotype of the T5KO males. We did not test the behavior of RE mice, but it is possible that the loss of a sex difference may lead to sex-dependent changes in behavior.

Oxytocin did not respond to inflammatory signals with the same intensity as vasopressin. Oxytocin tends to be anxiolytic and anti-inflammatory (Neumann and Slattery, 2016; Yuan et al., 2016b), and cytokines can modulate the transcription and production of oxytocin. This occurs in a conditional manner, based on the location of oxytocin and cytokines, as well as the individual (Wang et al., 2015). Thus, it is possible that we did not see much effect of inflammation on oxytocin expression due to the chronic nature of our models. Oxytocin may play more of a role in moderating immune activation during more acute insults but may exert less influence during chronic inflammation. Furthermore, it is possible that the microbiota models we used permanently altered the oxytocin system in locations or ways that were not investigated

in these experiments, or oxytocin may be acting to reduce the damage from chronic inflammation.

Oxytocin tends to reduce food intake, suggesting that the elevations in oxytocin immunoreactivity in GF mice may contribute to their obesity resistance (Bäckhed et al., 2007; Herisson et al., 2016; Spetter and Hallschmid, 2017; Spetter et al., 2018). Oxytocin is also involved in motivation, parental behavior, and addiction (Love, 2014; Yoshihara et al., 2017; Leong et al., 2018), suggesting that there may be many other reasons for the increase in oxytocin immunoreactivity in the GF mice that we did not test in these experiments. More investigation into the roles of oxytocin in the gut-brain axis are needed.

Taken together, gut-derived inflammation is associated with greater vasopressin immunoreactivity in SCN and PVN projection sites of the brain and contributes to disordered anxiety-like, depressive-like and social behaviors. Oxytocin may be playing a role in the increased sociability of T5KO mice but have less influence over anxiety-like and depressive-like behaviors in these models. Future experiments are needed to tease apart the exact actions each neuropeptide has in this axis.

4.2 Commentary on Behavioral Testing

Unfortunately for behavioral neuroscientists interested in translational implications of their work, rodent behavior is not a perfect analog of human behavior. Thus, it is difficult to produce and validate mouse models of psychiatric disorders, especially when these disorders have large cognitive components that are not testable in rodents. It is also difficult to say whether the artificial assays we use for anxiety-like and depressive-like behaviors are truly testing the behaviors we think they are. For

example, the marble burying test is used as a measure of anxiety-like behavior, repetitive behavior, and aversive behavior, among others, and is based on the idea that rodents bury items that are anxiogenic to them (Kedia and Chattarji, 2014; Sanathara et al., 2018; De Brouwer et al., 2019). However, Njung'e and Handley found that there was no habituation to the marbles on repeated testing, they did not avoid the marbles when given a choice of a marble-filled or empty arena, and administration of anxiogenic agents did not increase the number of marbles buried (Njung'e and Handley, 1991). Some use the marble burying test alone as a measure of anxiety-like behavior (Nardo et al., 2014; Gawali et al., 2016), and others, us included, use the marble burying test as part of a test battery for anxiety-like behaviors and measure more behaviors than just the number of marbles buried. It is imperative that if we are to use rodents to investigate the neural mechanisms of behavior in the context of developing therapies for psychiatric disorders, then we need to examine a wide variety of behaviors in numerous behavioral tests.

An intriguing way to gain an overall impression of a behavioral phenotype is to use multiple discriminant analysis to build a model that predicts future group inclusion in an unbiased manner (Fields et al., 2018a). This statistical method determines the relationship between each variable and each individual in the analysis, create functions that explain the variance between each data point, and identify which dependent variables are contributing most to each function (Cooley and Lohnes, 1971). We used discriminant analysis in Chapter 3 to gain a better understanding of the behavioral phenotype of T5KO mice and to see if there was a pattern to the behavior of T5KO-g mice that individual comparisons alone did not capture. We conclude that T5KO mice

show mild sickness behavior because the factors that contributed the most to separating the genotypes in the model were ones indicating lethargy, increased depressive-like behavior, and repetitive grooming. These behaviors are all dependent on activity level, which is a prime behavior affected during sickness behavior (Dantzer et al., 2008). We saw significant changes in anxiety-like behavior in T5KO mice, but the discriminant analysis suggested that those behaviors were more responsible for separating the sexes from one another, and not genotypes. This suggests that anxiety-like behavior may be a more important factor in sickness behavior in females or that T5KO affects anxiety-like behavior in males and females differently. While this analysis is not appropriate in all situations, it is a way to statistically mimic the way our brains identify patterns in behavior and may be a useful tool in further behavior research.

4.3 Pathways of microbiota-gut-brain-behavior communication

4.3.1 Microbiota to gut signaling

As mentioned in the introduction, there are a number of mechanisms through which microbiota can communicate with the gut, either to effect local change or to send signals to other organ systems like the brain. Briefly, microbiota reside in the gut in the thick, outer layer of mucus, which provides a good support for the development of biofilms and provides an energy source if the host's diet is insufficient (Russell et al., 2011a; Kelly et al., 2015). Underneath this layer is a relatively sterile, thin layer of mucus that is tightly adhered to the intestinal epithelial layer. The epithelial layer is composed of a number of cell types, including enteroendocrine cells (EECs) that can produce hormones, including serotonin, Paneth cells that host TLRs to sense bacterial penetration, and other cells that are involved in sampling the intestinal milieu to ensure

homeostasis (Samuel et al., 2008; Vaishnava et al., 2008; Haghikia et al., 2015; Yano et al., 2015). Microbiota can produce a number of metabolites like short chain fatty acids (SCFA), hormone precursors such as tryptophan, and other molecules like secondary bile acids that communicate with the EECs or the mucosal immune system (Wikoff et al., 2008; Tolhurst et al., 2012; Yano et al., 2015). SCFAs are mainly produced through the fermentation of dietary fiber the host cannot digest alone, and the less dietary fiber present in the diet, the less SCFAs produced (Topping and Clifton, 2001; Russell et al., 2011b). SCFAs mediate the production of 5-hydroxytryptamine (5-HT) by EECs, so the gut microbiota can directly influence the amount of 5-HT produced by the gut, which has downstream implications for neural 5-HT and serotonin production (Clarke et al., 2013; Yano et al., 2015).

GF mice, despite lacking a microbiota, still produce gut-derived signaling molecules, including 5-HT. Because they do not have microbiota to ferment indigestible starch, they do not have the microbiota regulation of tryptophan production, which affects the amount of 5-HT produced in the gut. There are conflicting reports on the amount of circulating tryptophan and 5-HT production in GF mice (Wikoff et al., 2008; Clarke et al., 2013), but it is clear that the lack of microbiota disrupts the serotonin system. Interestingly, when GF mice are colonized with microbiota, plasma 5-HT levels are normalized but hippocampal 5-HT is not (Clarke et al., 2013). These experiments suggest that the lack of microbiota affects the production of enteric hormones which has long-lasting effects on brain structure and function. 5-HT can regulate vasopressin release, such that administration of 5-HT rapidly increases plasma vasopressin release (Pérgola et al., 1993), and there is a significant body of literature examining the interplay

between 5-HT and vasopressin in aggression (Morrison and Melloni, 2014; Terranova et al., 2016). Thus, it is possible that the dysregulation of serotonin may have far-reaching effects on vasopressin and behavior.

T5KO mice have an increased bacterial load, thinner mucus layer, more adherent bacteria to the epithelial wall, and increased SCFA production, providing multiple avenues through which microbiota in this model can affect the gut (Carvalho et al., 2012b; Singh et al., 2015a). Chronic inflammation can result in increased permeability of the intestinal epithelium and is further exacerbated by the encroachment of microbiota on the epithelial layer in T5KO mice, resulting in either a stronger immune response or more translocation of bacterial products, like SCFA, across the membrane. While SCFA are typically considered to be beneficial, in T5KO mice they aggravate metabolic syndrome by traveling to the liver and contributing to insulin resistance when added to the diet (Singh et al., 2015). This suggests that elevated SCFA may disrupt other systems by modulating immune or metabolic activity, which may have downstream effects on the brain and behavior. More investigation into whether SCFA are elevated in the brain will further elucidate the roles that SCFA play in this pathway.

4.3.2 Gut to brain signaling

Similar to the number of ways microbiota can communicate with the gut, there is a myriad of pathways that transfer information from the microbiota to the brain. A primary path is through vagal activation. The vagus nerve innervates the heart, lungs and digestive tract and sends signals to the hypothalamus by way of the nucleus of the solitary tract (Chavan et al., 2017). The vagus nerve expresses innate immune receptors and responds mainly to endocrine or immune signals. Many actions of

microbiota on behavior are dependent on vagal nerve communication, as vagotomy abolished the anxiolytic effect of *Bifidobacterium longum* on mice with colitis and prevented the reduction in stress-induced depressive-like behavior in mice by *Lactobacillus rhamnosus* (Bercik et al., 2011; Bravo et al., 2011). However, other experiments still found behavior-moderating effects of probiotics in vagotomized mice, suggesting that there are other mechanisms at play (van der Kleij et al., 2008). It would be interesting to see if T5KO mice would still exhibit the same behavioral phenotype after vagotomy. One study found that the number of TLR5-expressing neurons is increased after bleomycin treatment to induce pulmonary fibrosis (Jung et al., 2018), suggesting that the vagus nerve is involved in some cases involving TLR5. It is also possible that activation of this neural pathway primes brain structures for the production and action of cytokines that propagate into the brain, essentially creating a mirror of the peripheral inflammation (Dantzer et al., 2008). Thus, it is possible that the brains of T5KO mice would be continuously primed for inflammation, leading to dysregulation of neuropeptide expression and resulting in the anxiogenic phenotype described above.

Another pathway is the humoral route, in which circulating cytokines or microbial proteins bind to receptors on cerebral endothelial cells (CEC) or circumventricular organs (CVO) to elicit a neural immune response (D'Mello and Swain, 2014). CEC make up the blood-brain barrier (BBB) and the tight junctions between CEC are affected by microbial products, similar to the intestinal epithelium (Braniste et al., 2014). For example, GF mice have a more permeable barrier than CC mice, likely due to deficits in bacterial products like SCFA (Braniste et al., 2014). Binding of receptors for TNF-a and IL-1B found on CEC activates NF-kB, which in turn induces production of second

messengers like prostaglandins or nitric oxide (Rivest et al., 2000). These second messenger systems can activate microglia and affect sickness behavior through expression in the hypothalamus and amygdala (Zhang and Rivest, 1999). There are also cytokine transporters in the BBB that allow systemic cytokines into the brain through volume diffusion (Banks, 2006). Monocytes can adhere to CEC and even transmigrate into the brain, providing another source of inflammation in the brain (Kerfoot et al., 2006; D'Mello et al., 2009). Cytokines or microbial products may enter the brain through CVO, which are brain regions that lack a functional BBB and include regions like area postrema and the median eminence (Dantzer et al., 2008). These regions produce c-fos mRNA, an immediate-early gene, in response to systemic TNF-a, and produce pro-inflammatory cytokines in the brain in response (Nadeau and Rivest, 1999). T5KO mice have increased systemic IL-1B and TNF-a, suggesting that these cytokines may be interacting with the brain through CEC or CVO (Carvalho et al., 2012c). More work needs to be done to elucidate whether the BBB is more permeable in T5KO mice and if T5KO mice show neuroinflammation.

4.3.3 Brain to Behavior Signaling

Inflammatory signaling from the periphery may affect other neural systems than vasopressin and oxytocin, like microglia. Microglia activation is associated with sickness and depressive-like behaviors, and inhibiting microglia ameliorated these behaviors (Henry et al., 2008; Corona et al., 2010; D'Mello et al., 2013). We showed that GF mice have reductions in microglial number and immunoreactivity that were unresponsive to colonization with microbiota, which corroborates our behavioral findings of reduced anxiety-like behaviors. Contrary to the social withdrawal associated with

activated microglia (Corona et al., 2010), we found that GF mice are less social than CC mice, suggesting that the deficits in social behavior may occur through a microglia-independent pathway.

We do not know the state of neuroinflammation in our T5KO mice. It is likely, based on their behavior profile and intestinal inflammation, that T5KO mice would have elevated cytokine expression, activated microglia, or another indication of neuroinflammation. There is some tangential evidence of neuroinflammation in T5KO mice. For example, T5KO mice show worse nerve regeneration after a crush injury and associated decreased BDNF expression (Hsieh et al., 2017). Other experiments show a protective effect of TLR5 activation in post-conditioning treatment after ischemic stroke, where sublethal hypoxia from TLR5 activation can protect against future stroke (Gu et al., 2016; Jeong et al., 2017). Furthermore, TLR5 expression is elevated in major depressive disorder and schizophrenia, and antidepressant and antipsychotic treatment, respectively, can normalize TLR5 expression (Hung et al., 2016; Kéri et al., 2016, 2017). These studies suggest that TLR5 activation induces inflammation in the brain and this activation can have behavioral consequences. It may be that T5KO will result in increased neuroinflammation, based on the peripheral inflammation in these mice, possibly through upregulation of other TLRs. Future experiments can be done to determine how TLR5 deficiency affects neuroinflammation.

4.3.4 Proposed Pathway

It is difficult to determine exactly how TLR5 deficiency results in changes to vasopressin and behavior without direct experimental evidence. However, we can predict a potential pathway. It is likely that the increased SCFA and intestinal pro-

inflammatory cytokines signal to the brain through the vagus nerve, which sends inflammatory signals to the hypothalamus, increasing vasopressin production. This increased vasopressin will be projected to other regions of the limbic system to increase anxiety-like behaviors, as well as increasing activation of the HPA axis. A secondary modulatory pathway may occur through translocation of bacteria through the gut epithelium, resulting in macrophage production of pro-inflammatory cytokines. These systemic cytokines may either breach the BBB through downregulation of tight-junctions or cytokine transporters. They may also bind to receptors on the CECs or CVOs to induce neuroinflammation, possibly through activation of microglia or production of cytokines in the brain.

Many more experiments are needed to test this pathway. A few possibilities would be to vagotomize T5KO mice to see if the behavioral effects are dependent on an intact vagus nerve, reducing intestinal inflammation by downregulating IL-1β and TNF-α using nanoparticle-delivered siRNA (Neuhaus et al., 2015) to determine if inflammation in the gut is part of this pathway, or selectively knocking down vasopressin in the PVN or SCN using AVP-Cre mice to determine if vasopressin is necessary for the behavioral phenotype of T5KO mice. These experiments could provide valuable information on how inflammatory signals transmit to the brain to effect behavior.

It is still unclear why the T5KO-microbiota treated mice did not show the same behavioral phenotype as the TLR5 knockouts. I predict that the inflammation was localized to the gut, which would explain the physiological markers of inflammation we saw but would also explain the lack of behavioral change. The T5KO-microbiota treated mice still have functioning TLR5 receptors in the gut, so they may act to attenuate the

inflammatory response. This may lead to less systemic pro-inflammatory cytokine production, and thus less of an impact on the brain. To determine where this pathway is interrupted, we need to do a careful examination of the microbiota, intestinal inflammation, systemic inflammation, neuroinflammation, and neuropeptide expression of the microbiota-treated mice. However, it is possible that this experimental paradigm is insufficient to test the effect that T5KO microbiota have on behavior. Because we colonized the parents of the tested animals, it is likely that the microbiota composition shifted close to that of the WT microbiota-treated mice. We will be able to determine if this is the case once we receive the results of microbiota sequencing from these animals.

If we wanted to more directly test whether T5KO microbiota impacts the behavior of WT mice, we could either colonize WT GF mice at birth or at weaning and assess their behavior in adulthood. We chose not to do this initially due to the feasibility constraints of recolonizing at birth, and we wanted the microbiota-transplanted mice to have microbiota for their entire lives. Recolonizing at weaning may be a good choice, because T5KO microbiota transplantation stabilize better when administered later in life, and there does not seem to be a developmental effect of T5KO microbiota (Fulde et al., 2018).

4.4 Implications for Human Health

4.4.1 Comorbidity between gastrointestinal and CNS disorders

Dysbiosis of the microbiota can have important implications for human health. A rapidly accumulating body of evidence shows that microbiota composition can influence the etiology and progression of neurological and psychiatric disorders, such as anxiety

disorders, major depressive disorder, and autism spectrum disorders (Dinan and Cryan, 2016; Lach et al., 2018). For example, anxiety disorders have been linked to increased inflammation (Felger, 2018) and are often comorbid with gastrointestinal symptoms, suggesting a role for the gut microbiota in the expression of these disorders (Powell et al., 2017). Specifically, patients with generalized anxiety disorder had lower levels of Faecalibacterium, Eubacterium rectale, Lachnospira, Butyricicoccus, and Sutterella, all genera that produce SCFA (Jiang et al., 2018; van de Wouw et al., 2018). This suggests that patients with anxiety disorders may have increased gut permeability, due to deficits in the protective SCFA. Furthermore, patients with anxiety disorders have higher levels of pro-inflammatory cytokines (Pitsavos et al., 2006; O'Donovan et al., 2010; Duivis et al., 2013), suggesting that dysbiosis of the gut can lead to increased inflammation, and subsequently anxiety disorders.

Patients with major depressive disorder have been shown to have increased microbial diversity, specifically increases in *Bacteriodetes*, *Proteobacteria*, and *Actinobacteria*, with decreases in *Firmicutes* (Jiang et al., 2015), whereas another group found a decrease in microbial diversity (Kelly et al., 2016). In the largest study to date, depressed patients had decreased levels of the *Coprococcus* and *Dialister* genera, and butyrate-producing bacteria were associated with higher qualities of life (Valles-Colomer et al., 2019). The inconsistent pattern of results suggests more research is needed to understand the role that microbiota play in major depressive disorders (Dinan and Cryan, 2019). Perhaps more important than the bacterial composition is the inflammatory potential of the microbiota, as inflammation is a key contributor to depression for some individuals (Derry et al., 2015). Interestingly, cytokine-induced

sickness behavior strongly resembles depression, and antidepressants can alleviate some components of sickness behavior (Yirmiya et al., 1999). Cytokine-induced sickness behavior may result in depressive-like behavior through reduction of tryptophan due to increased activation of the tryptophan metabolizer, indoleamine 2,3 dioxygenase (IDO) (Wirleitner et al., 2003; Ruhé et al., 2007). IDO breaks down tryptophan into kynurenine, which can be transported across the BBB, then either into 3-hydroxykynurenine (3-HK) and quinolinic acid (QA), or kynurenic acid (KA). 3-HK and QA is preferentially produced by microglia and tend to cause oxidative stress, whereas KA is produced by astrocytes and can be neuroprotective (Cervenka et al., 2017). Thus, activation of microglia may trigger this pathway to produce more 3-HK and QA, increasing the likelihood of depression. Furthermore, increased breakdown of tryptophan by IDO can result in less tryptophan available for conversion to serotonin in the brain, which may contribute to a depressive phenotype (Delgado, 2000). Finally, major depressive disorder is more common in patients receiving immunotherapy and ones that have chronic inflammatory disorders (Raison et al., 2006).

Children with ASD show an altered gut microbiota composition, such as an increase in Clostridium, Bacteriodetes, and Lactobacillus, compared to higher numbers of Firmicutes in neurotypical controls, suggesting an imbalance of beneficial bacteria (Finegold et al., 2010; Adams et al., 2011). Treatment of children with ASD with the antibiotic vancomycin, which preferentially affects the gut microbiome, alleviates autism symptoms (Finegold et al., 2002). Furthermore, children with ASD have a correlation between the severity of autism and gastrointestinal symptoms (Mulle et al., 2013). While these measures are correlative and it is highly likely that external factors

can contribute to these measures, as the diets of children with ASD are often different from neurotypical controls, there is a high likelihood that the microbiota plays a significant role in the onset and expression of ASD.

Inflammatory gastrointestinal disorders and metabolic syndrome have high comorbidity with ASD, anxiety, and mood disorders (Härter et al., 2003; Adams et al., 2011; de Sousa Rodrigues et al., 2017; Tang et al., 2017; Fowlie et al., 2018; Penninx and Lange, 2018b), but it is still unclear the direction of causality in these cases. Using mouse models like T5KO mice to mimic the relationship between obesity, inflammation, and psychiatric disorders will bring us closer to understanding and treating these disorders.

4.4.2 Dietary Considerations

An interesting consequence of the recent popularity of investigating the gut microbiota is a refocus on diet as a way to improve human health. We are discovering that dietary additives like emulsifiers and artificial sugars, added to provide a specific texture to food or reduce consumption of sugars, both promote intestinal inflammation and weight gain, as well as alter the gut microbiota (Chassaing et al., 2015; Palmnäs et al., 2014; Pepino, 2015; Suez et al., 2014; Swithers, Martin, Clark, Laboy, & Davidson, 2010). In the case of emulsifiers, we recently found that the detrimental effects of emulsifier consumption can increase anxiety-like behavior and decrease preference for social novelty in a sex-dependent manner, suggesting that the chemicals we add to our food may directly influence our behavior (Holder et al., 2019).

Our dietary patterns are correlated with human health, with gut microbiota composition acting as a mediator. The gut microbiota is shaped by diet, and changes to

the diet can result in rapid alterations to microbiota composition (David et al., 2014; Sheflin et al., 2017), so it is unsurprising that our dietary choices can greatly affect our health. For example, diets high in fat can facilitate LPS translocation across the epithelial barrier, which can generate chronic inflammation, but diets high in dietary fiber tend to increase microbiota diversity (Caesar et al., 2015; Tap et al., 2015). We are beginning to understand the health benefits of some popular diets, like the ketogenic and gluten-free diets. In fact, we are finding that the ketogenic diet decreases bacterial diversity due to the lack of carbohydrates, but actually increased beneficial strains of bacteria, including *Lactobacillus* (Ma et al., 2018). A ketogenic diet can also alleviate some of the symptoms of ASDs and can manage epilepsy in children (Newell et al., 2016; Olson et al., 2018). Food intake patterns can affect the gut microbiota as well, such as in intermittent fasting. Intermittent fasting increases bacterial diversity, reduces IL-17 producing T cells, decreases obesity, and can ameliorate the disease progression of multiple sclerosis in a mouse model (Li et al., 2017a; Cignarella et al., 2018). Investigating the effect dietary choices have on the microbiome, and human health, can provide us with ways to take preventative steps against disease progression.

4.5 Conclusions

Our organ systems communicate with each other and the environment in a vast number of ways that we are only beginning to uncover. From the gut microbiota, the gut, the immune system, the endocrine system, to the brain and behavior, a delicate balance of homeostasis is maintained. Perturbations anywhere along this system can have long-lasting, deleterious impacts on human health. Future experiments are

needed to mechanistically identify interactions between these systems in order to develop more effective treatments for both gastrointestinal and psychiatric disorders.

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Zhang L, Doroshenko P, Cao XY, Irfan N, Coderre E, Kolaj M, Renaud LP (2005) Vasopressin induces depolarization and state-dependent firing patterns in rat thalamic paraventricular nucleus neurons in vitro. Am J Physiol Integr Comp Physiol 290:R1226–R1232.

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APPENDIX: CURRICULUM VITAE

CURRICULUM VITAE

EDUCATION

Master of Science, Neuroscience, Fall 2015

Georgia State University, Atlanta, GA (2012-2015)

Bachelor of Arts, Psychology (Magna cum laude; Honors fellow)

Siena College, Loudonville, NY (2007-2012)

Bachelor of Sciences, Biology (Magna cum laude; Honors fellow)

Siena College, Loudonville, NY (2007-2012)

Honors Thesis: The role of androgens in the development of sexual orientation

EXPERIENCE

Graduate Research Assistant

Georgia State University, Atlanta, GA (2012-present)

-Rotated in the labs of Dr. Aras Petrulis and Dr. Elliott Albers

-Currently in the lab of Dr. Geert de Vries

Chemist Aide (Chemical Safety Office and Media Lab)

Wadsworth Center, NYS Department of Health, Albany, NY (2010-2012)

-Produced solutions and agar for research labs

-Assisted in lab inspections, hazardous waste disposal and chemical safety training modules

SAT Tutor

Huntington Learning Center (2012)

-Assessed students' weaknesses in their SAT performance and taught them techniques to improve their SAT or ACT score

Research Assistant (Research Experience for Undergraduates program)

Duke University, Durham, NC (2009)

-Worked in the lab of Dr. Christina Williams investigating the organizational and activational effects of hormones on adult neurogenesis

FELLOWSHIPS & GRANTS

- Center for Neuromics Student Grant, Georgia State University (2014)
- 2CI Neurogenomics Fellowship, Georgia State University (2012-present)
- Presidential Scholar Fellowship, Siena College (2007-2012)

PUBLICATIONS

1. **Peters NV,** Holder MK, Chassaing B, Paul MJ, Whylings J, Gewirtz AT, and de Vries GJ (in prep). Microbiota are necessary for proper neural vasopressin and oxytocin development.

2. **Peters NV,** Chassaing B, Holder MK, Whylings J, Gewirtz AT, and de Vries GJ (in prep). Sex-dependent anxiogenic and depressive-like phenotype in TLR5 knockout mice associated with changes in neural vasopressin and oxytocin.
3. Holder MK, **Peters NV,** Fields CT, and de Vries GJ (in prep). Sex differences for life. *Frontiers in Neuroendocrinology*.
4. Whylings J, Rigney N, **Peters NV**, de Vries GJ, and Petrulis A (in revision). The role of BNST vasopressin cells in anxiety-like, depressive-like and social behavior following sickness in male and female mice. *Brain, Behavior, and Immunity.*
5. Tran HQ, Mills RH, **Peters NV,** Holder MK, de Vries GJ, Knight R, Chassaing B, Gonzalez DJ, Gewirtz AT (in press). Associations of the fecal microbial proteome composition and proneness to diet-induced obesity, *Molecular &Cellular Proteomics.* https://doi.org/10.1074/mcp.RA119.001623
6. Holder MK, **Peters NV,** Whylings J, Fields CT, Gewirtz AT, Chassaing B and de Vries GJ (2019). Dietary emulsifiers affect the intestinal microbiota and alter anxiety and social behaviors in a sex-dependent manner, *Scientific Reports* **9**(172). DOI: 10.1038/s41598-018-36890-3
7. Paul MJ, **Peters NV**, Holder MK, Kim AM, Whylings JJ, Terranova JI, and de Vries GJ (2016). Atypical social development in vasopressin-deficient Brattleboro rats. *eNeuro,* 3**(2)**. DOI: 10.1523/ENEURO.0150-15.2016
8. de Vries GJ, Fields CT, **Peters NV**, Whylings JJ, and Paul MJ (2014). Sensitive periods for hormonal programming of the brain. *Current Topics in Behavioral Neurosciences,* 16, 79-108. DOI: 10.1007/7854_2014_286

ORAL PRESENTATIONS

1. Neuroscience Institute Breakfast Lecture. "Chronic inflammation and behavior: investigating the role of microbiota." Atlanta, GA (2018).
2. ION Program. "Gut microbiota and behavior." Emory University, Atlanta GA (2018).
3. Neurogenomics Forum. "Decoding the microbiome." Atlanta, GA (2017).
4. Neuroscience Institute Breakfast Lecture. "Gut feelings: How microbiota influence brain and behavior." Atlanta, GA (2016).
5. Neuroscience Institute Breakfast Lecture. "Sex differences in anxiety across social development." Atlanta, GA (2014).

POSTER PRESENTATIONS & PUBLISHED ABSTRACTS

1. **Peters NV,** Chassaing B, Holder MK, Whylings J, Gewirtz AJ, and de Vries GJ. Knockout of TLR5 in mice results in sex-dependent changes to neural vasopressin. Organization for the Study of Sex Differences, Montreal, QC, Canada, 2017.
2. **Peters NV**, Chassaing B, Holder MK, Whylings JJ, Gewirtz AJ, and de Vries GJ. Sex effects of TLR5 knockout on physiology and behavior. Keystone Symposia, Keystone, CO, 2017.
3. **Peters NV,** Paul MJ, Chassaing B, Dunn J, Gewirtz AT, and de Vries GJ. Increased oxytocin immunoreactivity in male and female germ-free Swiss-Webster mice. Society for Neuroscience, San Diego, CA, 2016.

4. **Peters NV**, Chassaing B, Holder MK, Whylings JJ, Gewirtz AJ, and de Vries GJ. Sex effects of TLR5 knockout on physiology and behavior. Organization for the Study of Sex Differences. Philadelphia, PA, 2016.
5. Holder MK, **Peters NV,** Castillo-Ruiz A, Mosley MD, Chassaing B, Gewirtz AT, Forger NG, de Vries GJ. Development of microglia of germ-free and conventionally colonized mice. Society for Neuroscience, Chicago, IL, 2015.
6. **Peters NV,** Paul MJ, Chassaing B, Gewirtz AJ, and de Vries GJ. Microbiota impact vasopressin immunoreactivity in adult Swiss-Webster mice in a sex-dependent manner. Society for Behavioral Neuroendocrinology, Pacific Grove, CA, 2015.
7. Paul MJ, **Peters NV,** Holder MK, Whylings J, Badeau C and de Vries GJ. Decreased number and mean frequency of ultrasonic vocalizations in juvenile Brattleboro rats. Society for Neuroscience, Washington, D.C., 2014.
8. **Peters NV,** Paul MJ, Chassaing B, Gewirtz AJ, and de Vries GJ. Microbiome impacts vasopressin immunoreactivity in juvenile Swiss-Webster mice. Society for Neuroscience, Washington, DC, 2014.
9. **Peters NV,** Paul MJ, Rhaney CN, Cooke BM, and de Vries GJ. Sex differences in behavioral states across pre-juvenile and juvenile development. Organization for the Study of Sex Differences, Minneapolis, MN, 2014.
10. Paul MJ, **Peters NV**, Kim AM, Shah CR, Probst CK, and de Vries GJ. Null mutation in the vasopressin gene eliminates sex differences in the development of social play in rats. Society for Behavioral Neuroendocrinology, Atlanta GA, 2013.

11. **Peters NV**. Effect of Androgen Hormones on Sexual Orientation Development. Association for Psychological Science, Chicago, IL, 2012.
12. Angstadt J, Simone A, and **Peters NV**. Effects of riluzole on cell DE-3 of the medicinal leech: Evidence that persistent sodium current contributes to post-inhibitory rebound responses and bursting activity induced by calcium channel blockers. Society for Neuroscience, Washington D.C., 2011.
13. **Peters NV**. Effect of Physiological Arousal on Perceived Attraction. Association for Psychological Science, Washington, D. C., 2011.

TEACHING & MENTORSHIP

Instructor of Record

- Introduction to Drugs and Behavior, PSYC 2050, Georgia State University (2018)

Teaching Assistantships

- Drugs and the Nervous System, NEUR 4150, Georgia State University (2018)
    - Responsible for all grading and gave lecture on antidepressants
- Drugs and the Nervous System, NEUR 4150, Georgia State University (2017)
    - Responsible for all grading, helped professor to develop class activities, and gave lectures on anxiolytics and antidepressants
- Drugs and the Nervous System, NEUR 4150, Georgia State University (2016)
    - Responsible for grading all of the quizzes given, performed the lectures on anxiolytics
- Cellular and Molecular Neuroscience, NEUR 4010, Georgia State University (2015)

- Developed a primary literature comprehension series, led class discussions, and graded primary literature assignments

Tutoring Experience

- Undergraduate Writing and research Skills for Biology Students, Siena College (2009-2011)
    - Led small group workshops, led peer-mentoring groups, graded scientific papers
- Higher Education Opportunity Program Tutoring, Siena College (2009)
    - Individually tutored students in chemistry, biology, philosophy, and statistics

Pedagogy Training

- General Pedagogy for Future College Teachers workshop
    - Provided instruction on how to develop courses, assignments, and evaluations, how to work with varying types of students, and how to develop a teaching philosophy
- Research Mentoring Training
- Using Constructive Criticism

Invited Lectures

- Intro to Drugs and Behavior, Georgia State University, “Depressants” (Spring 2018)
- Neuroimmunology Seminar, Georgia State University, “Microbiota and relevance to neuroimmunology” (Spring 2014)

Mentorship

- Daniel Teusher (2017- 2019)
- Julian Dunn, Brains & Behavior Undergraduate Scholar (2016-2017)
- Kristen Jackson, ION Scholar (2015)
- Beth Allyn, ION Scholar (2014)
- Krishna Mehta, Brains & Behavior Undergraduate Scholar (2013-2016)
- Christina Rhaney, ION Scholar (2013-2014)

  *ION is an NIH-funded educational summer research program for high school teachers and students.

PROFESSIONAL SERVICE

- Organization for the Study of Sex Differences, Trainee Social Coordinator (2017-present)
- Neuroscience Graduate Student Association, co-president (2015-2016)
- Professional Development for Neuroscientists Seminar, grant reviewer (2015)
- Neuroscience Institute Graduate Representative Committee, member (2014-present)
- Neuroscience Institute Breakfast Lecture, organizer (2014-2015)
- Neuroscience Institute Recruitment Weekend, assistant (2013-2016)

COMMUNITY SERVICE

- Hey, You Touched My Brain! Workshop, Atlanta Science Festival (2019)
- Organization for the Study of Sex Differences Annual Meeting Program Booklet Developer (2018)

- Assistant to Organization for the Study of Sex Differences (September 2016)
- Gathered information on membership to help plan next year's meeting
- Informal Education and Training Mentor for the Atlanta's NET/work Undergraduate Research Program (January 2016)
- Build-a-Brain Workshop, Discovery Day, Atlanta Science Festival (March 2016)
- Build-a-Brain Workshop, Science at Hand Day at Fernbank Museum (November 2014 and 2015)

PROFESSIONAL MEMBERSHIP

- Society for Neuroscience (2012-present)
- Society for Behavioral Neuroendocrinology (2013-present)
- Organization for the Study of Sex Differences (2013-present)
- Psi Chi Honor Society (2011-present)
- Pi Sigma Gamma Sigma Biology Honor Society (2011- present)
- Delta Epsilon Sigma Honor Society (2011-present)

RESEARCH INTERESTS

- Neuroendocrinology
- Sex differences
- Behavioral Development
- Microbiota
- Social & Anxiety Behaviors

BENCH SKILLS

- Immunohistochemistry
- Enzyme-linked immunosorbent assay (ELISA)
- Microscopy (Bright field, fluorescent, confocal)
- Polymerase chain reaction (PCR)
- Behavioral testing and scoring
- Surgery
- Cannula implantation
- Single injection neurosurgery
- Gonadectomy
- Osmotic mini-pump implantation
- Histology (including tissue removal and processing)
- Statistical analysis