UNIVERSITY OF CALIFORNIA, SAN DIEGO

Nod1/Nod2 Receptors Modulate the Microbiota-Gut-Brain Axis

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by

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The thesis of Melinda A. Schneider is approved and is acceptable in quality and form for electronic and microfilm publication:

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University of California, San Diego

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DEDICATION

I dedicate this thesis to Cindy and Doug for their love, encouragement and support, and to Jenny who can always make me laugh.

EPIGRAPH

Dancing in all its forms cannot be excluded from the curriculum of all noble education; dancing with the feet, with ideas, with words, and, need I add that one must also be able to dance with the pen?

Nietzsche

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ABSTRACT OF THE THESIS

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Melinda A. Schneider

Master of Science in Biology

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Professor Melanie Gareau, Chair

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The association between intestinal diseases, mood disorders and cognitive deficits is increasingly recognized. Previous studies from our lab suggest that chronic intestinal diseases, such as infection with an enteric pathogen, in combination with an acute psychological stressor (water avoidance stress [WAS]), can induce anxiety-like behavior and memory defects in mice. In our current study,

we hypothesized that nucleotide binding oligomerization domain (Nod) receptors, as important modulators of homeostasis between the host immune system and the gut microbiota, are integral regulators of the microbiota-gut-brain (MGB) axis. Using Nod1/Nod2 double knockout (NodDKO) mice, we performed behavioral testing, qPCR analysis of stool bacterial DNA, and serum corticosterone analysis. Compared to wild type (WT) controls (C57BL/6), NodDKO mice displayed anxiety-like behaviors (light/dark box test, $p<0.001$) and deficits in recognition memory (novel object recognition [NOR] test, $p<0.001$) following exposure to a single session of WAS. WT and NodDKO mice differed in the composition of their microbiota in 4 of 7 bacterial groups studied by qPCR, indicating the presence of intestinal dysbiosis in NodDKO mice. Baseline elevated levels of serum corticosterone were also observed in NodDKO mice. To evaluate a potential role for the microbiota in mediating these effects given the presence of dysbiosis in NodDKO mice, WT and NodDKO mice were co-housed at weaning and behavior assessed in adulthood. Co-housing indicated that Nod1/Nod2 receptors are important for recognition memory and anxiety-like behavior, with defects remaining despite partial microbiota normalization. Interestingly, co-housing also ameliorated the WAS-induced spike in corticosterone levels in co-housed WT and NodDKO mice. Therefore, our study indicates that Nod receptors serve as key signaling molecules, potentially acting via the hypothalamic–pituitary–adrenal (HPA) axis, to modulate the MGB axis in mice.

I.

INTRODUCTION

Resident intestinal bacteria have an increasingly appreciated influence on overall human health and disease, both within and outside the gastrointestinal tract. There is a growing body of evidence linking changes in the composition of the gut microbiota with impaired cognitive capacities and altered mood in both human studies and pre-clinical models. (Mayer, Knight, Mazmanian, Cryan, & Tillisch, 2014) An understanding of the interplay of factors inducing modifications in the gut microbiota, and subsequently influencing cognitive function, is in its early infancy, although the field is expanding rapidly. More fundamental to this topic, however, is the investigation of the mechanisms of bidirectional communication between the central nervous system and the microbiota that mediates these cognitive deficits.

Interplay between the immune system, both innate and adaptive, is thought to be central to the cognitive connection with the gut and microbiota. Therefore, the aim of our study was to identify the role of innate immune receptors, Nod 1 and Nod 2, on the microbiota-gut-brain (MGB) axis. These pattern recognition receptors (PRRs) are important in maintaining homeostasis with activation resulting in cell signaling cascades leading to gene transcription, and pro-inflammatory pathways including downstream cytokine production.

1.1 *The Gut Microbiota and Health*

The human gut is colonized by 10^{14} microbes – more than 10 times the number of cells in the human body. (Clark & Coopersmith, 2007) These microbes

consist of more than 1,000 species, most of which are anaerobic strains of unknown species. (Honda & Takeda, 2009) In order of prevalence in the gut, the main bacterial phyla are Firmicutes*, Bacteroidetes, Actinobacteria, Proteobacteria, Verrocomicrobia* and *Fusobacteria.* (Zhang et al., 2015) Usually, the gut bacteria interact with the host in a commensal manner, providing nutrients, synthesizing vitamin K, aiding in the digestion of cellulose, promoting angiogenesis, and modulating enteric nerve function. (Hill & Artis, 2010; Mueller & Macpherson, 2006) For example, *Bacteriodetes* and Firmicutes are largely responsible for the breakdown of undigested food. (Tsuji, Suzuki, Kinoshita, & Fagarasan, 2008) In return, the host provides a protected and nutrient rich environment for the commensal bacteria to reside and thrive. (Tsuji et al., 2008) In conditions of well - being, the microbiota and host interact symbiotically, conferring health to the host.

The gut microbes also play a large role in host defense. A healthy microbiota promotes healthy epithelial barrier function, and protects the host from noxious antigens and pathogenic organisms via the "colonization effect" that they provide. (Ulluwishewa et al., 2011) One study, which utilized the CD4+CD62L transfer model of colitis, showed that animals receiving effector lymphocytes isolated from animals raised in an axenic, or germ free (GF) environment, developed colitis earlier on compared to mice reconstituted with effector lymphocytes isolated from conventionally-housed mice. (Strauch et al., 2005) Furthermore, mice receiving regulatory lymphocytes from GF mice were not able to abrogate the colitis, compared to the recovery seen following reconstitution

with regulatory lymphocytes from conventionally housed mice. (Strauch et al., 2005) These results indicate that bacterial antigens may be crucial for the healthy generation and expansion of regulatory T cells, implicating the microbiota with an emerging role in the regulation of the immune system.

Increasingly, the gut microbiota community structure is influenced by numerous factors, including diet and environmental exposures, consumption of beneficial probiotic bacteria and administration of antibiotics. In addition, clinical immune pathologies including obesity, diabetes, inflammatory bowel disease (IBD; ulcerative colitis [UC] and Crohn's disease [CD]) are also associated with patients displaying dysbiosis in their microbiota. (Jostins et al., 2012) Although no single intestinal microbiota profile exists that is deemed a gold standard of health, certain ratios of bacterial phyla are implicated with a more healthy state of the gut microbiome. For example, *Bacteroidetes* and Firmicutes are the two dominant bacterial divisions and their relative abundance may mediate metabolic changes associated with lean or obese phenotypes. (Turnbaugh et al., 2006) Furthermore, the metabolic changes associated with an "obese-microbiota" are transferable to lean mice. (Turnbaugh et al., 2006) In general, an increased diversity of the microbiota is generally associated with better overall health.

1.2 *The Microbiota-Gut-Brain Axis*

The complex connection between the commensal intestinal microbiota and behavior, including cognitive function, has received increasing attention in recent

years. Since reduced cognitive function is a common comorbidity of numerous clinical pathologies including, but not limited to, metabolic diseases, intestinal diseases and mood disorders, a role for the microbiota in modulating these diseases could have important clinical implications. (Jostins et al., 2012) Colloquially known as "the forgotten organ," the intestinal microbiota has been well-accepted in recent years as an important regulator of behavior, including anxiety and depression. (Borre, Moloney, Clarke, Dinan, & Cryan, 2014; Mayer, Knight, Mazmanian, Cryan, & Tillisch, 2014; Tillisch, 2014) However, the influence of these gut bacterial populations is likely to extend to other capacities of the brain, including cognition, via the MGB axis. Research investigating the exciting interplay between factors modulating the gut microbiota and their subsequent influence on cognitive function is rapidly expanding.

One of the early studies designed to assess the effect of dietary modifications on the composition of the microbiota, and subsequent behavioral changes, was a preliminary animal study in which mice were fed a diet of 50% lean ground-beef-enriched chow. Mice fed this diet not only displayed a significantly more diverse microbiota, but performed better in behavioral cognition tests (i.e. the hole-board open field test) compared to control animals fed standard chow. (W. Li, Dowd, Scurlock, Acosta-Martinez, & Lyte, 2009) Similarly, mice fed a diet high in fat and sugar, or “Western diet” was associated with cognitive dysfunction in the form of deficiencies in learning, memory, and executive function, as well as behavioral changes including depression, anxiety, and dementia. (Pyndt Jørgensen

et al., 2014) Studies have shown that such a high fat diet (HFD) can induce a shift in the ratios of *Bacteriodetes* to Firmicutes populations by reducing the former and increasing the latter, as well as decreasing the overall diversity of microbes. (Khan, Raine, Donovan, & Hillman, 2014; Turnbaugh et al., 2006) In a groundbreaking preclinical study in mice, *Bruce-Keller et al.* transferred the cecal and colonic contents of donor mice that were fed a HFD to non-obese wild type (WT; C57BL/6) mice. This resulted in the synchronized transfer of the donor (HFD and obese) mouse's cognitive deficits to the recipient wild type mouse. (Bruce-Keller et al., 2014) This study sought to identify the effect of HFD-induced dysbiosis on cognitive deficits, including memory, executive function, and stereotypical behavior (marble burying), in the absence of obesity and nutritional deficits. Upon confirming, through ribosomal DNA phylogenetics that the protocol successfully transferred the bacterial communities among mice, producing distinct microbiotas, behavioral tests were performed to evaluate the cognitive function of these mice. The elevated plus maze (EPM) and the open field test both reflect anxiety-like behavior of the animal by measuring the total time spent in the center of the EPM, or the time spent in the corners of an open well-lit field. The longer the period of time the mouse spends in these protected regions of the test space correlates with an increasing anxiety level. Additionally, a video-based fear conditioning system was used to test fear-associated memory, by testing the animal’s ability to remember associations between a tone and unpleasant foot shock. Mice colonized with a microbiota from an unhealthy-diet donor showed elevated anxiety-like

behavior and decreased memory as compared to mice colonized with microbiota from non-obese donors. (Bruce-Keller et al., 2014) As measured by western blot, specific synapse-associated proteins, including phosphorylated synapsin 1, were significantly reduced in the medial prefrontal cortex of HFD-shaped microbiota mice, suggesting decreased synapse density and plasticity. It was also noted that mice with the unhealthy-diet-shaped microbiota have increased intestinal permeability, as measured by quantification of tight junction proteins. (Bruce-Keller et al., 2014) In combination with elevated serum endotoxin levels and lymphocyte expression of TLR4, this permeability deficit could be evidence of induction of alterations to the MGB axis via activation of pro-inflammatory immune responses. (Bruce-Keller et al., 2014) This study provided the first definitive findings that the microbiota-induced changes following HFD are sufficient to disrupt brain physiology and function in wild-type recipient mice, even in the absence of altered diet, obesity or metabolic syndrome.

The complex relationship between the microbiota and the brain is further substantiated by studies that have demonstrated that HFD-induced dysbiosis can result in anxiety-like and depressive behaviors in mice subjected to chronic social stress. (Finger, Dinan, & Cryan, 2011) Although the mechanisms by which the gut microbes affect behavior and mediate stress, anxiety and depression are not well understood, the physiological changes seen in the *Bruce- Keller* study suggest multiple possible mechanisms could be involved, including immune activation or direct interactions with neural function. Activation of the immune system and

altered neural activity are both associated with nearly every neurological or psychiatric disorder. (Zhou & Foster, 2015) Thus, this study suggests that unhealthy, HFD-induced changes in the microbiota may increase the risk of neurological conditions associated with inflammation including, but not limited to, autoimmune diseases, Alzheimer's disease and autism spectrum disorder (ASD). Maintaining a healthy diet, and in the future, therapeutic manipulation of the microbiota to achieve that of a healthy non-obese person, could be effective in mitigating prevalence and severity of neuropsychiatric disorders. (Bruce-Keller et al., 2014)

IBD, irritable bowel syndrome (IBS), and metabolic disorders including diabetes and obesity, are associated with intestinal dysbiosis and frequently manifest with psychological disturbances, including impaired learning and memory, elevated anxiety and depression. (Gareau, 2014; Koboziev, Reinoso Webb, Furr, & Grisham, 2014) These findings suggest that a potential correlation exists between psychiatric disorders and the gut microbiota via the MGB axis. Although the mechanism by which altered microbiota-induced cognitive changes is not well characterized and likely multifactorial, several neuronal, humoral, and hormonal factors have been linked to this process. (Koboziev et al., 2014) Recent findings in the literature suggests that modifications of the microbiota may prevent or treat a variety of psychiatric syndromes, often with comorbidities in both the gastrointestinal tract and neuropsychological function, serving as further evidence of the key role the microbiota in modulating the gut-brain axis.

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is important in response to exposure to physiological stressors, and increasingly it has been shown to be fundamentally involved in the MGB axis. Exposure to acute psychological stress induces HPA-axis activation and cortisol (corticosterone in rodents) production by the adrenal glands. In the gastrointestinal tract, activation of the HPA axis following stress results in altered intestinal barrier function, (Gareau, Silva, & Perdue, 2008) and altered microbiota composition, (Konturek, Brzozowski, & Konturek, 2011). It has also been demonstrated to trigger anomalies in behavior including mood disorders and cognitive defects following infection with an enteric pathogen. (Gareau et al., 2011) Therefore, stress-induced activation of the HPA axis may be an intrinsic regulator of the MGB axis.

The comorbidities seen in psychiatric and gastrointestinal illness implicate the MGB axis as a significant mediator in disease pathogenesis. Psychiatric diseases, including depression, schizophrenia and ASD-behaviors, as well as systemic diseases, including metabolic disorder, IBD and IBS, respond to dietary and probiotic interventions in pre-clinical rodent studies. Furthermore, these studies find that altering the gut microbiota may lead to cognitive improvements, suggesting potential clinical applications for treating common cognitive and mood disorders, as well as intestinal disease with psychological comorbidities. Future studies that decipher mechanistic underpinnings of the MGB axis will expand clinical applications and lead to the identification of specific targets for improved cognitive treatment outcomes.

1.3 *Nod1/Nod2 Receptors: Intracellular Bacterial Sensors*

The immune system is elemental in regulating homeostasis and health of the host, protecting it from harmful stimuli including bacteria and viruses. The resident micro-organisms that inhabit the lumen of the gut are in very close proximity to the single layer of epithelial cells that separate the host from the outside environment. Although the microbiota is mainly composed of healthy symbionts, potentially pathogenic organisms can invade, creating a need for a well-developed immune defense to maintain this healthy host-microbiota homeostasis.

Nod-like receptors (NLRs) are innate immune regulators that differentiate beneficial symbiont organisms from pathogenic ones and aid in eliciting the proper protective host response to these pathogens. (Rescigno, 2011) NLRs serve as intracellular PRRs that are critically involved in maintaining mucosal immune defense by recognizing and binding to damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). Detection of these DAMPS and PAMPS by NLR induces activation of either the nuclear factor kappaB (NF-kB) mediated mitogen activated protein kinase (MAPK) signaling pathway, or by formation of inflammasomes. (Claes, Zhou, & Philpott, 2015) As a class of PRRs, inflammasomes activate a pro-inflammatory signaling cascade involving caspase activation and recruitment domain (CARD). (Hornung & Latz, 2010) Activation of NLR signaling pathways leads to downstream signaling cascades and immune responses including cytokine production, important for responding to a potential luminal threat to the host.

Of critical interest to this current study are Nod1 and Nod2 receptors which each sense unique components of bacterial peptidoglycan (PGN) and are part of the NLRC (NLRs containing CARD) subfamily of receptors. Nod1 and Nod2 each recognize unique molecular motifs of intracellular PGN breakdown products: Nod1 senses meso-diaminopimelic acid (ie. DAP) found mainly in Gram-negative bacteria (Girardin, Boneca, Carneiro, et al., 2003) , whereas Nod2 recognizes muramyl dipeptide (MDP) found ubiquitously in both Gram-negative and Gram-positive bacteria (Girardin, Boneca, Viala, et al., 2003). Nod1 is expressed in most cell types and tissues, including low levels in murine brains (Franchi, Warner, Viani, & Nuñez, 2009; Liu, Han, & Leng, 2014). Nod2 is expressed in monocytes, lymphocytes, intestinal Paneth cells and, at comparatively higher levels than Nod1, in the pituitary and hippocampus of the brain. (Correa-de-Santana et al., 2009; H. Li et al., 2010; Liu et al., 2014; Ogura, 2003) The expression of Nod receptors, particularly in the brain, implicates them as potentially prime mediators of the MGB axis.

Recent research has expanded the Nod1 and Nod2 receptors' roles in sensing bacteria. Although these cytoplasmic receptors lack transmembrane domains, Nod1 and Nod 2 receptors may be recruited to the plasma membrane during infections. (Barnich, Aguirre, Reinecker, Xavier, & Podolsky, 2005; Kufer, Kremmer, Adam, Philpott, & Sansonetti, 2008) In addition, interactions with the cytoskeleton were shown to regulate nonspecific responses or aid in efficient recruitment of receptors. (Keestra et al., 2013; Legrand-Poels et al., 2007)

Therefore, in addition to sensing PGN, Nod1/Nod2 are also sensors of cytoskeleton reorganization induced by microbiota shifts during pathogenic invasion. The implications of the bacterial sensing capabilities of NLRs continue to be investigated – from shaping the development of the microbiota to aiding in the defense against intestinal infections and maintaining gut-homeostasis. (Claes et al., 2015) Our current study provides evidence that expands the potential role of NLRs to mediating behavioral and cognition changes as well.

1.4 Nod1/Nod2 Role in Intestinal Disease

Accumulating evidence suggests that chronic intestinal diseases can often involve an aberrant host response to the commensal intestinal microbiota. (Halme et al., 2006) Nod 1 and Nod 2 receptors have been implicated in chronic inflammatory diseases, including those in the gut, with several genome-wide studies identifying Nod2 as the most common susceptibility factor for the development of CD. (Hugot et al., 2001) CD patients often have loss-of-function mutations in Nod2 receptors resulting in unresponsiveness to MDP. (Netea et al., 2005) Nod receptors drive a protective inflammatory response through receptor-interacting protein 2 (RIP2) and NF-kB that stimulates the production of antimicrobial peptides and mucin at the epithelium, which enforce a physical gap between the epithelium and the gut microbiota. Non-functional Nod receptors, as in CD, lead to the breakdown of this protective inflammatory response and result in a loss of barrier function. Compensatory inflammatory pathways respond,

resulting in chronic inflammation characteristic of CD. (Philpott, Sorbara, Robertson, Croitoru, & Girardin, 2014) Although Nod1 receptors were also initially hypothesized as risk factors for IBD, later studies have not confirmed this correlation. (McGovern et al., 2005)

Both Nod1 and Nod2 have been found to be integral to responding to enteric infection as demonstrated in mouse models. For example, Nod1-deficient mice are more susceptible to *Helicobacter pylori* and *Clostridium difficile* infections (Hasegawa et al., 2011; Viala et al., 2004) and Nod 2-deficient mice carry a higher bacterial burden upon *Listeria monocytogenes* infection. (Opitz et al., 2005) In addition, Nod1 and Nod2 double deficient (NodDKO) mice develop exacerbated colitis upon dextran sodium sulfate (DSS) treatment and have an elevated risk of developing DSS-induced colorectal cancer. (Barreau et al., 2007; Chen, Shaw, Redondo, & Núñez, 2008; Couturier-Maillard et al., 2013) However, upon characterization of healthy NodDKO mice in comparison to WT mice, no significant differences in body or organ weight were reported, as well as comparable leukocyte count. Other biochemical analyses of the NodDKO mice, including lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase (organ damage markers), amylase (marker of pancreatitis) and plasma albumin (oncotic pressure regulator), were not significantly different between WT and NodDKO mice. (Stroo et al., 2012) Furthermore, vascular permeability was unchanged, however intestinal permeability was found to be elevated, suggesting the presence of a gut phenotype. (Stroo et al., 2012) Thus, while NodDKO mice are less resilient

when challenged with enteric infection and disease compared to WT, baseline inflammation and sickness symptoms are minimal.

The ileal, cecal and fecal microbiota profiles of Nod1$^{-/-}$ and Nod2$^{-/-}$ mice have also been individually characterized via qPCR and no differences in microbiota population structures were detected in comparison to WT mice. (Natividad et al., 2012; Robertson et al.) However, another study contradicts this finding, revealing that Nod2 knockouts may have an altered microbiota. (Couturier-Maillard et al., 2013) The current theory is that Nod1 and Nod2 receptors function synergistically to tune the appropriate inflammatory responses. In a recent study, NodDKO mice were found to have barrier dysfunction, increased intestinal permeability and worsened response to intestinal injury in comparison to WT mice. (Natividad et al., 2012) This group sought to determine whether modulating the intestinal microbiota of NodDKO mice via colonization with a microbiota devoid of pathogens and opportunistic bacteria (Altered Schaedler flora (ASF)) or probiotic administration could improve this basal altered intestinal barrier function and response to intestinal injury. When comparing ASF colonized Nod1 $^{+/-}$/Nod2$^{+/-}$ knockdown mice to NodDKO mice, no difference in response to DSS-induced colitis were observed, suggesting that "colitogenic" bacteria are also required for increased susceptibility to DSS-induced colitis in NodDKO mice. (Natividad et al., 2012) While probiotic administration to NodDKO mice improved the outcome of DSS-induced colitis, the authors also found that neither ASF colonization nor probiotic administration were effective in improving baseline

intestinal permeability defects in NodDKO mice. (Natividad et al., 2012) These results suggest that Nod1 and Nod2 receptors have a direct impact on permeability. (Natividad et al., 2012) The authors ultimately concluded that the intestinal microbiota influences colitis severity in NodDKO mice. (Natividad et al., 2012) This study highlights the complex interactions between the microbiota and genetic factors that are associated with chronic intestinal diseases and establishes the use of the NodDKO mouse for further investigation of the genetic factors that may regulate the microbiota, gut and subsequently the brain.

Therefore, to investigate the connection between the Nod1 and Nod2 receptors and the microbiota, gut and brain, our group studied behavior and cognition in NodDKO mice. We hypothesized that Nod1 and Nod2 receptors would play an important role in shaping the behavior and cognition of mice via the MGB-axis and HPA-axis. Understanding the role of NLRs in the MGB-axis may elucidate the foundation of the comorbid behavioral abnormalities seen in IBD patients, providing potential strategies for future therapies.

II.

MATERIALS AND METHODS

2.1 ***Animals:***

Nod1/Nod2 double knockout (NodDKO) mice (C57BL/6 background) were compared to WT C57BL/6 mice throughout the study. Both male and female mice, ages 6-8 weeks, were utilized. Mice were housed in cages lined with chip bedding and had free access to food and water 24 hours each day. The vivarium lighting schedule allowed 12 hours of light and 12 hours of darkness each day. Animals were bred and housed in a University of California, San Diego animal facility and all behavioral testing was performed in a biosafety cabinet. All behavioral tests were performed between the hours of 9am and 6pm and all procedures and protocols were reviewed and approved by IACUC at the University of California, San Diego. Mice were kindly provided by Dr. Dana Philpott, University of Toronto and subsequently bred in house.

2.2 ***Co-housing:***

Upon weaning at 3-4 weeks of age, two NodDKO mice were housed in the same cage as two similarly aged, sex-matched WT mice. The mice remained co-housed until testing and sacrifice at 6-8 weeks of age. This co-housing strategy aimed to normalize the gut-microbiota profiles between the WT and NodDKO mice by unifying environmental exposures. (Hufeldt, Nielsen, Vogensen, Midtvedt, & Hansen, 2010)

2.3 *Water Avoidance Stress (WAS):*

Prior to behavioral testing, a subgroup of both conventionally-housed and co-housed experimental groups were subjected to the acute psychological stressor WAS. Mice were placed on small platforms (inverted 50ml beakers) in a clean, standard housing cage filled with approximately 2 cm of room temperature water for 1 hour. After completion of stress, mice were returned to their home cage and allowed to rest for 5-10 minutes prior to behavioral testing. (Mönnikes, Schmidt, & Taché, 1993) *(Refer to **Illustration 1** for schematized WAS setup)*

2.4 *Novel Object Recognition (NOR) Test:*

Prior to the NOR test, mice were subjected to 1 hour of habituation in individual cages inside a lit biosafety cabinet. The NOR test is comprised of three phases - training, resting, and testing – to evaluate the mouse's recognition memory capacity. (Antunes & Biala, 2012) *(Refer to **Illustration 3** for NOR test paradigm)*

Training Phase:

Two novel objects, a round napkin ring (object A) and a star shaped cookie-cutter (object B) were placed in opposite corners of the cage. The mice were allowed to freely explore each object for 5 minutes while being video-recorded. Upon conclusion of the training phase, the objects were removed from the cage.

The training phase serves to expose the mouse to two novel objects and confirm that the mouse has no preference for exploring one object over the other.

Resting Phase:

During the resting phase between training and testing, the animal spends 15 minutes in the cage without any objects. The resting phase serves as a time barrier during which the animal may process the information of the two objects leading to recognition memory formation or forgetting.

Testing Phase:

During the testing phase, object B, the star shaped cookie-cutter from the testing phase, and a novel object C, a different uniquely shaped napkin ring, are placed in the cage in opposite corners. Again the mouse is allowed to investigate the objects for 5 minutes under video surveillance.

NOR Test Data Analysis:

The video recordings were later scored and the number of times the mouse sniffed or physically contacted each object (an investigation) was recorded. The results are expressed as an exploration ratio calculated from the testing phase data:

$$Exploration\ ratio = [\frac{(\#\ of\ investigations\ of\ object\ C)}{(\#\ of\ investigations\ of\ object\ C) + (\#\ of\ investigations\ of\ object\ B)}] \times 100\%$$

This calculation quantifies the mouse's preference of exploring one object more than the other during the testing phase – an indication of a mouse's recognition memory capability. An exploration ratio of 50% indicates that the mouse explored both objects equally during the testing phase, while an exploration ratio greater than 50% indicates that the mouse investigates the novel object C more than the familiar object B, indicating high memory recall for the familiar object. (Antunes & Biala, 2012)

2.5 ***Light/Dark Box Test:***

To measure anxiety-like behavior, mice were placed in a cage that allows for free exploration of darkened and well-lit regions of the light/dark box setup (**Illustration 1)**. The test proceeded for ten minutes, during which the behavior of the mouse in the light/dark box was video recorded. These videos were later scored for the total time the mouse spent in the lit half of the cage, the absence of which is an indicator of anxiety-like behavior, and the number of times the mouse transitioned from the dark region to enter the lighted area of the cage, a reflection of a mouse's activity level. Mice demonstrating a higher degree of anxiety-like behavior spend more time in the dark portion of the cage. (Bourin & Hascoët, 2003)

2.6 *Study Design:*

Both conventionally and co-housed mice were subjected to one of two tracks for behavioral testing: either stressed conditions or unstressed conditions.

Unstressed Condition:

On the day of behavioral testing, mice were retrieved from the vivarium in their home cages and placed in the biosafety cabinet for an acclimatization period (30-60min). Next, the mice individually underwent the 10 minute light/dark box behavioral test, followed by a habituation phase (1hr) in which the mouse was placed in an individual clean cage with bedding in the biosafety cabinet to rest. After habituation, the NOR test was conducted in the mouse's individual cage.

Stressed Condition:

Mice were allowed to acclimatize in their home cages in the biosafety cabinet (30-60min) and then were placed in individual cages with clean bedding for a habituation period (1hr). After the habituation, mice were subjected to WAS (1hr). Following WAS, mice were placed back in their individual habituated cages for 5 minutes prior to initiating the NOR test followed by the light dark box test. WAS induces HPA axis activation and may exacerbate behavioral defects. (Gareau et al., 2011)

Sacrifice and Collection

After all behavior testing was completed, the mice were sacrificed by CO_2 inhalation and cervical dislocation. At sacrifice, blood was collected via cardiac puncture, centrifuged at 5000RPM for 10 minutes for plasma separation and stored at -80°C for later hormonal analysis. Whole brains and colonic samples were harvested and stored in 10% formalin for two days prior to tissue processing for histology. Fecal samples were also collected and stored at -80°C for future microbiota analysis via qPCR.

2.7 *qPCR Analysis of the Microbiota:*

Colonic fecal samples were collected at sacrifice and frozen at -80°C. Bacterial DNA from stool was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) using a silica membrane spin-column based purification technique. Isolated DNA was amplified via qPCR using SYBR and primer sets (listed in ***Table 1***) that target 16s rRNA of bacterial groups covering approximately 60% of the gut microbiota including, Eubacteria (all bacteria; universal primer), *E. rectale,* segmented filamentous bacteria (SFB)*, Bacillus*, *Lactobacillus*, Enterobacteriaceae, *Bacteroides*, and Firmicutes. The composition of the NodDKO microbiota was compared to that of WT mice and these particular primers were previously validated to exclude cross reactivity. (Barman et al., 2008) The PCR cycling conditions were as follows: 1 cycle for 2 minutes at 50°C , 1 cycle for 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C followed by 1 minute at 60°C. A melt

curve was performed for quality control. Results are presented as a percentage expression of each bacterial group relative to total *Eubacteria*.

2.8 *Corticosterone:*

Cardiac puncture was used to collect blood at sacrifice and samples were spun down in serum separator tubes at 4°C. To measure corticosterone levels in serum, a commercial enzyme immune assay (EIA) kit (Enzo Life Sciences) was utilized and samples were read using a fluorescent plate reader (UC San Diego GI Center Core) giving serum corticosterone concentration in units of ng/mL.

2.9 Statistics

Results are expressed as means +/- standard error of the mean (SEM). Outliers, calculated as greater than +/- 1.5 standard deviations from the mean, were excluded from the data set. An unpaired t-test with Welch's correction or a one way ANOVA (Sidak's multiple comparison test) were performed as appropriate using Prism 6 GraphPad (San Diego, CA)

III.

RESULTS

3.1 NodDKO mice have stress-induced behavioral and memory defects

We first sought to investigate potential behavioral deficits of NodDKO mice. NodDKO mice demonstrated normal baseline anxiety-like behavior and recognition memory compared to WT controls ***(Figure 1).*** In contrast, NodDKO mice exhibit increased stress-induced anxiety-like behavior as compared to stressed WT mice, as measured by the reduced time stressed NodDKO mice spent in the light ***(Figure 1A).*** In addition, NodDKO mice exhibit stress-induced recognition memory defects as evidenced by their decreased exploration ratio in the NOR test compared to WT controls (***Figure 1B*****).** NodDKO mice did, however, have a reduced baseline of exploratory behavior compared to WT mice as evidenced by the reduced number of transitions between the light and dark compartment, which was not further enhanced following exposure to WAS (***Figure 1C***).

3.2 NodDKO mice have intestinal dysbiosis that is restored via co-housing

NodDKO mice differed in 4 of the 7 bacterial groups quantified in this study including Enterobacteriacaea (***Figure 2A***), *Bacillus* (***Figure 2B***), Segmented Filamentous Bacteria (***Figure 2C***) and *Lactobacillus* (***Figure 2D***) compared to WT controls. *Bacteroides,* Firmicutes and *Eubacterium Rectale* were present at the same levels in both WT and NodDKO mice and were unaffected by co-housing (***Figures 2E-2G***).

Enterobacteriaceae and SFB were elevated in conventionally housed NodDKO mice as compared to WT mice, but co-housing normalized the level to the baseline of conventionally housed WT mice (***Figure 2A, 2C***). *Bacillus* was also elevated in NodDKO mice, but co-housing led to the normalization in WT mice to match the elevated NodDKO levels (***Figure 2B***). *Lactobacillus* was elevated in NodDKO mice, and co-housing did not ameliorate the difference between WT and NodDKO mice as levels remained elevated in co-housed NodDKO mice as compared to co-housed WT mice (***Figure 2C***).

3.3 Changes in behavior in NodDKO mice were not restored upon co-housing with WT mice

To evaluate the effect of the microbiota on behavior in NodDKO mice, behavioral tests were performed with co-housed NodDKO and WT mice. Behavioral deficits were demonstrated in the co-housed NodDKO mice in the light/dark box test compared to WT cage-mates and were unchanged in comparison to conventionally housed NodDKO mice. Co-housed NodDKO mice retained the stress-induced anxiety-like behavioral deficit, in which the mice transitioned from light to dark sides of the cage less frequently than WT mice (***Figure 3A***) and spent less time in the light as compared to stressed WT mice (***Figure 3B***). Similarly, in the NOR test, NodDKO mice displayed stress-induced deficits in recognition memory compared to unstressed controls (***Figure 3C***). Surprisingly, the co-housing paradigm resulted in the transfer of the stressed

recognition memory defect from the NodDKO mice to co-housed WT mice, as evidenced by the reduction in exploration ratio in stressed co-housed WT mice compared to unstressed co-housed controls. (***Figure 3C***).

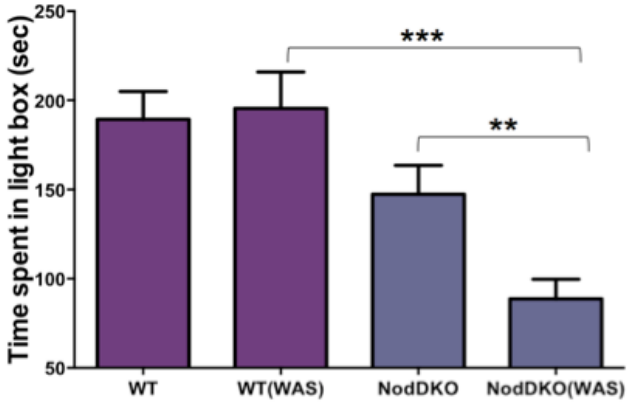
3.4 Elevated serum corticosterone levels indicate increased activation of HPA axis in NodDKO mice.

WAS is known to potently and reproducibly induce activation of the HPA axis. (Gareau et al., 2011) Consequently, as expected, both the WT and NodDKO mice exposed to WAS had elevated serum corticosterone levels (***Figure 4A***). NodDKO mice also had elevated baseline levels of serum corticosterone as compared to WT controls (***Figure 4A***). Following co-housing, however, baseline NodDKO levels were reduced and stress-induced changes in serum corticosterone levels were abolished in both WT and NodDKO groups, leading to an absence of activation following WAS exposure. (***Figure 4B***)

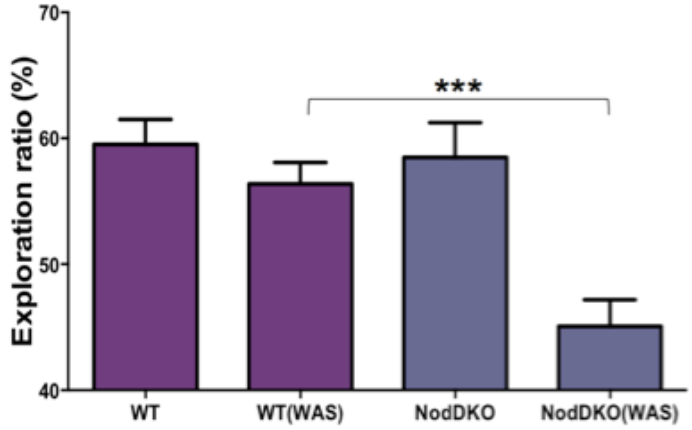
Figure 1: ***NodDKO mice display anxiety-like behavior and cognitive deficits compared to C57BL/6 controls.*** In WT mice, exposure to a single session of water avoidance stress (WAS) had no impact on behavior, including exploratory, anxiety-like and recognition). Similarly, no baseline changes were observed in anxiety-like behavior, but stressed NodDKO mice spent less time in light compared to stressed WT mice (***A;*** P<0.001) indicating elevated anxiety-like behavior in stressed NodDKO mice. Unstressed NodDKO mice perform comparably to unstressed WT mice in the NOR test (***B***). Stressed NodDKO mice, however, have a decreased exploration ratio during the testing phase of NOR test as compared to stressed WT mice (***B;*** p<0.001) indicating disrupted recognition memory in stressed NodDKO mice. NodDKO mice transition between the light and dark less frequently than WT mice (***C;*** p<0.001) indicating basal decreased exploratory behavior, which was not further enhance by exposure to WAS (***C***; p<0.0001).

(**p<0.01, ***p<0.001, ****p<0.0001, unpaired t-test, n=12-24).

A. Light/Dark Box Time in Light (Anxiety-Like Behavior)



B. Novel Object Recognition Test (Recognition Memory)



C. Light/Dark Box Transitions (Exploratory Behavior)

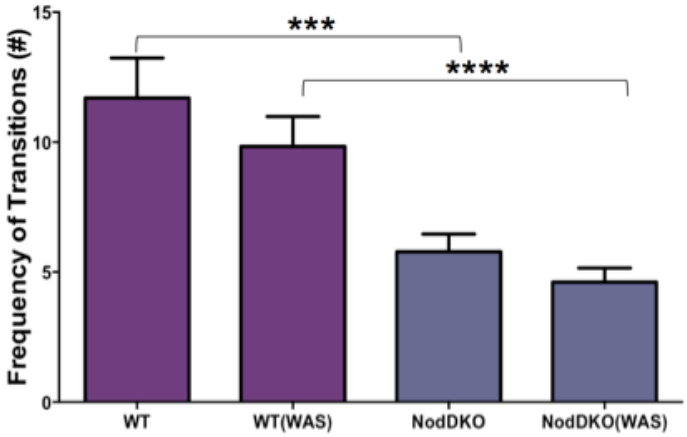
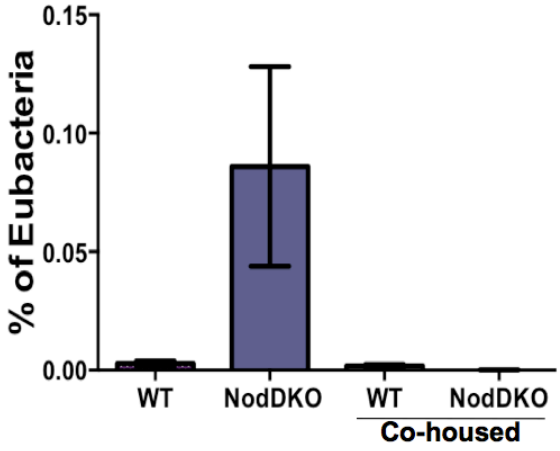


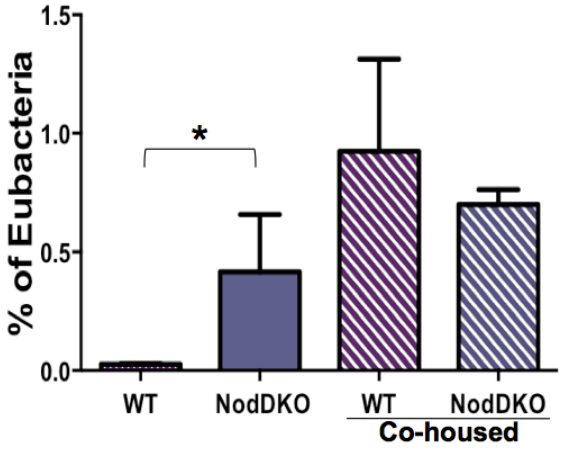
Figure 2: ***Dysbiosis was observed in NodDKO mice compared to C57BL/6 controls, which was normalized by co-housing strategies.***
The microbiota of WT and NodDKO mice was assessed by qPCR. WT and NodDKO mice differed in their composition of their microbiota in 4 of the 7 bacterial groups examined (Enterobacteriaceae (***A***), *Bacillus* (***B***), Segmented Filamentous Bacteria (SFB) (***C***), and *Lactobacillus* (***D***)). The remaining 3 bacterial groups examined (*Bacteriodes* (***E***), Firmicutes (***F***), and *E. rectale* (***G***)) had similar populations in both WT and NodDKO mice. All bacterial groups were normalized with co-housing, except *Lactobacillus* (***D***).

(*p<0.05, **P<0.01, unpaired t-test, n=5-15)

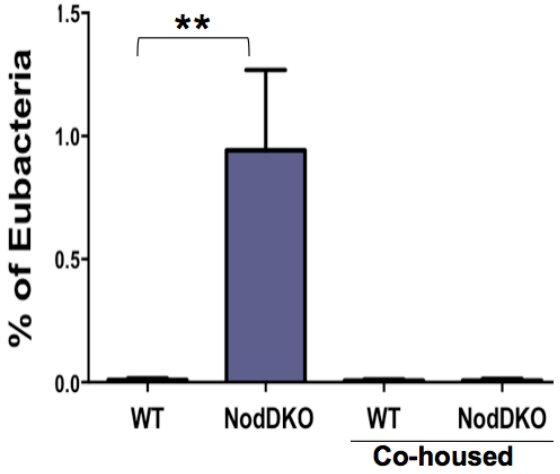
A. Enterobacteriaceae



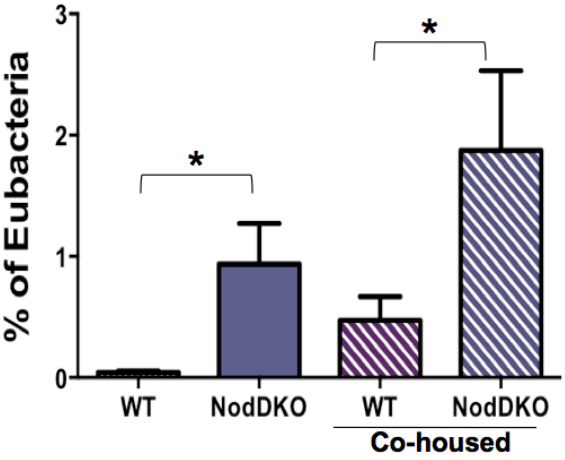
B. *Bacillus*



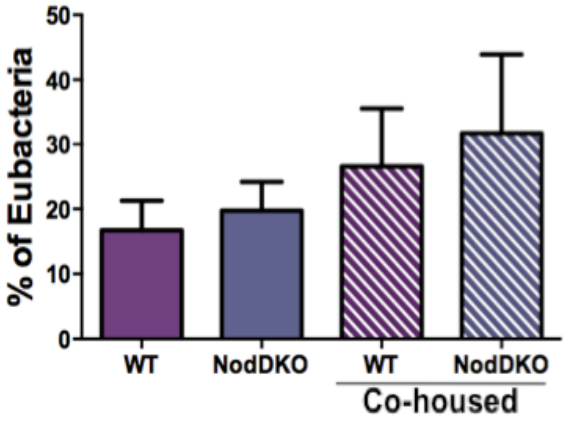
C. Segmented Filamentous Bacteria



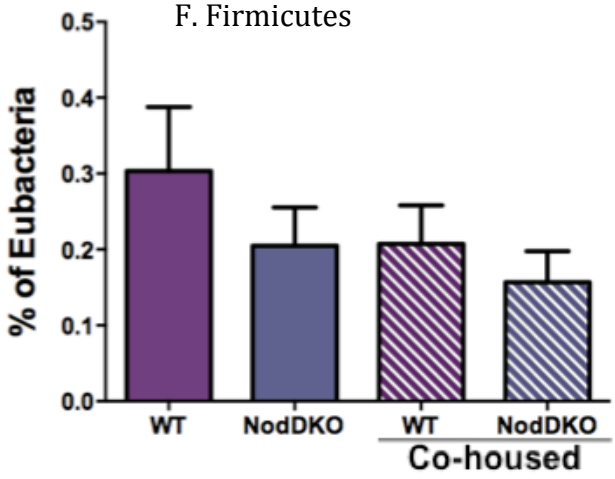
D. *Lactobacillus*



E. *Bacteriodes*



F. Firmicutes



G. *E. rectale*

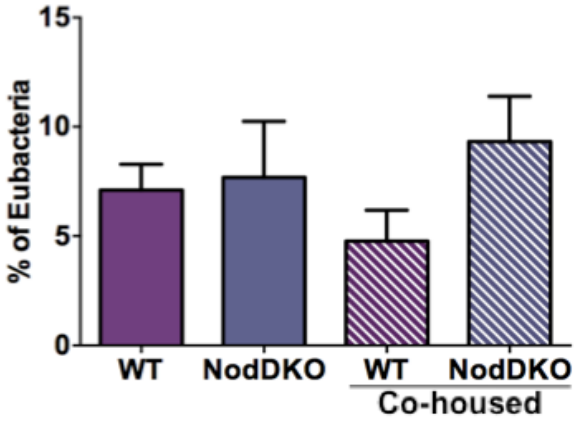


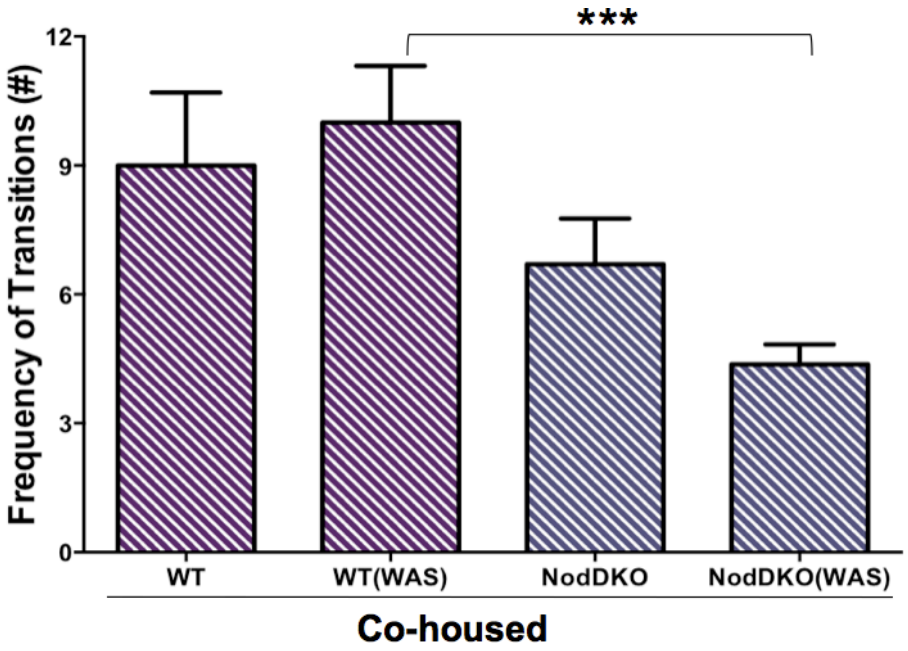
Figure 2 continued

Figure 3: ***Changes in behavior in NodDKO mice were not restored upon co-housing with C57BL/6 mice***

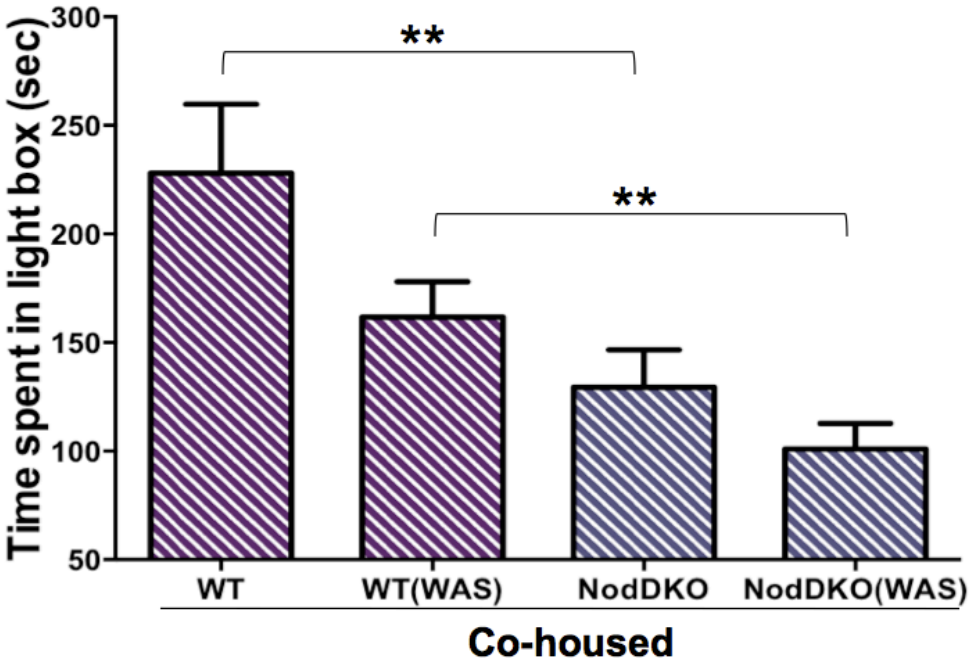
Co-housing did not ameliorate anxiety-like behavior (***A & B***) or recognition memory demonstrated in NodDKO mice compared to WT controls (***C***). Co-housing of mice beginning at weaning transferred the stress-induced NodDKO memory impairment phenotype to WT mice (***C***).

(*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, unpaired t-test, n=9-20).

A. Light/Dark Box Transitions (Exploratory Behavior)



B. Light/Dark Box Time in Light (Anxiety-Like Behavior)



C. Novel Object Recognition Test (Recognition Memory)

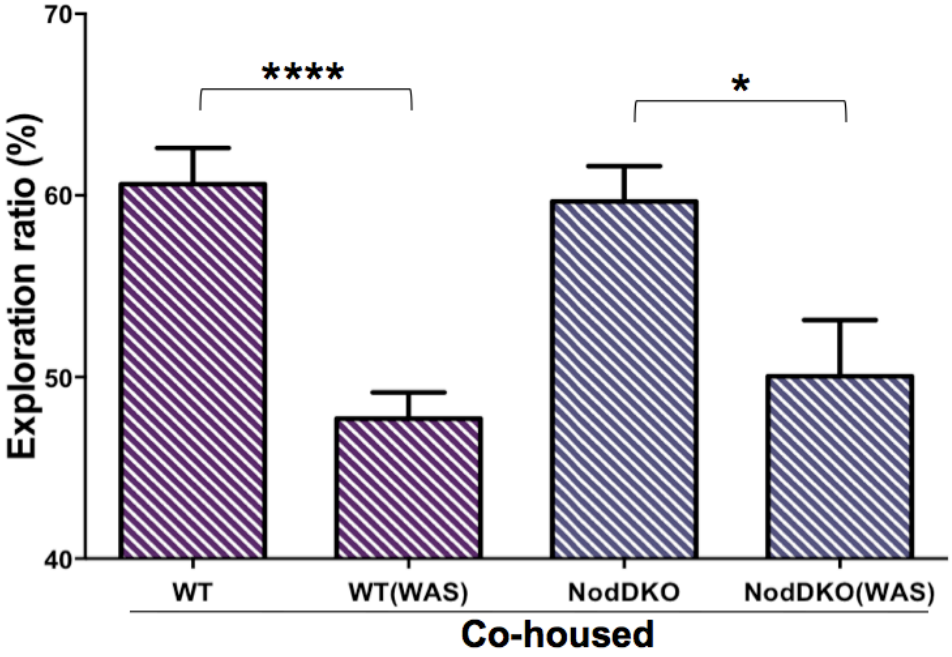
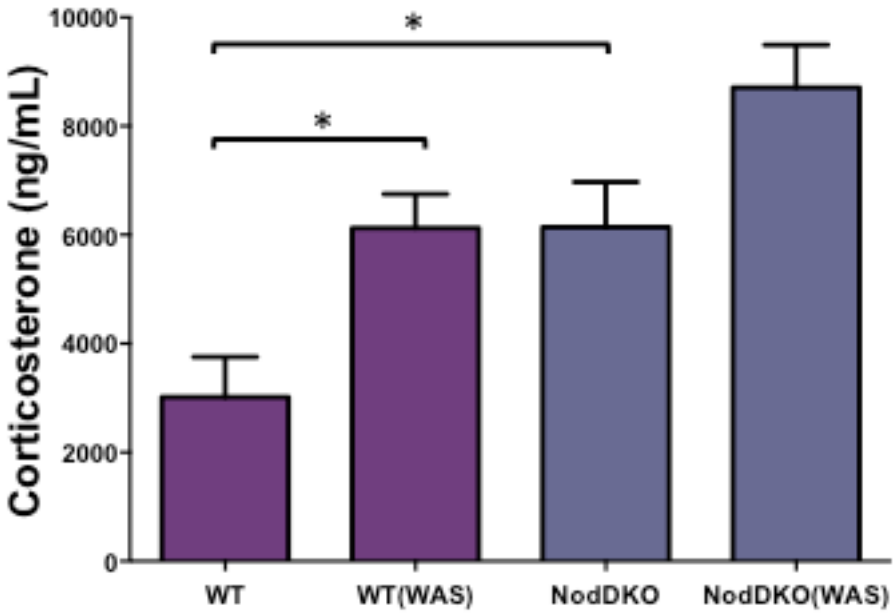


Figure 3 continued

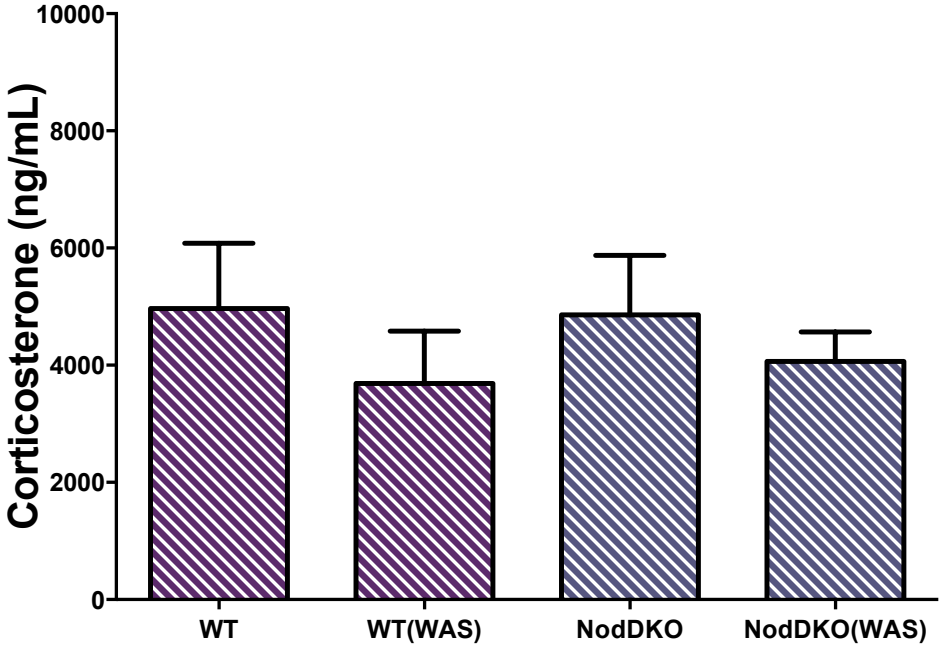
Figure 4: ***Elevated serum corticosterone level indicates increased activation of the HPA axis in NodDKO mice.*** WAS induces HPA axis activation in both NodDKO and WT mice (***A***). NodDKO mice exhibit elevated baseline levels of HPA activation (***A***; $p<0.05$), as compared to WT controls. Co-housing abolished baseline changes in serum corticosterone levels in NodDKO mice and reduced levels in all groups following exposure to WAS (***B***).

(*$p<0.05$, ***$p<0.001$, unpaired t-test n=9-10).

A. Serum corticosterone levels in conventionally housed mice



B. Serum corticosterone levels in co-housed mice



IV.

DISCUSSION

This study demonstrates that innate immune Nod receptors are integral modulators of the MGB-axis. Our results indicate that genetically knocking out both Nod1 and Nod2 receptors leads to stress-induced anxiety-like behavior and impaired recognition memory. As indicated by elevated serum corticosterone levels, NodDKO mice have a hyperactive stress response of the HPA axis. We propose that Nod-like receptors may modify the MGB axis via the HPA axis. Furthermore, NodDKO mice, as compared to WT mice, have an altered intestinal microbiota composition, which can be partially restored upon co-housing with WT mice. Despite a partial restoration of the microbiota and attenuation of the HPA-axis, co-housed NodDKO mice still displayed stress-induced behavioral deficits. Together, these results suggest that Nod-like receptors play an important role in the MGB axis, in part via the HPA-axis.

Changes in mood and cognition are increasingly being associated with gastrointestinal diseases, where the immune system plays a key role in physiology and homeostasis. Anxiety-like behavior can be assessed in mice via the light/dark box test, which measures the time spent in the lighted compartment of the box (Bourin & Hascoët, 2003). Reduced time spent in the light compartment is reflective of anxiety-like behavior. Similarly, the NOR test is effective in evaluating recognition memory in mice by measuring the ability of a mouse to discriminate a novel from a known object. (Antunes & Biala, 2012) Baseline behavioral testing revealed normal anxiety-like behavior and recognition memory in NodDKO mice compared to WT controls, in the presence of reduction in overall exploratory

behavior. In contrast, exposure to a single session of psychological stress, using WAS, resulted in NodDKO mice displaying decreased exploratory behavior, elevated anxiety-like behavior and reduced recognition memory. Previous studies from our group using *C. rodentium* infection in mice demonstrated that underlying behavioral defects were uncovered following WAS exposure prior to behavior testing. (Gareau et al., 2011) In our current study, behavioral deficits in the NodDKO mice were similarly uncovered by exposure to WAS, with baseline behaviors similar to WT mice. We also previously demonstrated that the behavioral defects induced by WAS and *C. rodentium* infection could be ameliorated with probiotic treatment. (Gareau et al., 2011) Thus, it was hypothesized that the NodDKO mice may have altered microbiota populations, or the presence of intestinal dysbiosis, as compared to WT mice, which may be contributing to the behavioral and cognitive defects we observed.

qPCR is a useful method by which the composition of the microbiota can be characterized. (Rinttilä, Kassinen, Malinen, Krogius, & Palva, 2004) This leads to enumeration of more species than traditional culturing without the high error rate seen with early sequencing analysis platforms. (Rinttilä et al., 2004) The fecal microbiota of NodDKO mice was analyzed by qPCR and compared to WT mice. Using this particular primer set, approximately 50-60% of the overall microbiota is enumerated. (Barman et al., 2008) We found that NodDKO mice differ significantly from WT control mice in their microbiota in 4 of 7 bacterial groups examined (Enterobacteriaceae*, Bacillus,* SFB and *Lactobacillus*). Elevated levels of

Enterobacteriaceae are associated with major depressive disorder in humans, according to one study of 46 patients, (Jiang et al., 2015), which correlates with the generalized dysbiosis associated with altered behavior and cognition seen in our previous studies. Hence we hypothesized that the differing microbiota was in part responsible for the elevated anxiety and reduced memory seen in NodDKO mice and sought to further test this through co-housing studies.

In order to normalize the microbiota between the WT and NodDKO mice, mice were co-housed starting at weaning and thereby exposed to identical environmental factors. Co-housing mice in this manner has previously been shown to unify the microbiome composition between cage-mates, regardless of genotype; because mice are coprophagic, the potential for transfer of gut microbiota via the fecal-oral route is high. (Garrett et al., 2007; Ridaura et al., 2013) Additionally, the microbiota is known to be more susceptible to long-term changes in composition at weaning than in adulthood. (Friswell et al., 2010) Co-housing of mice was successful in making the microbiota of the WT and NodDKO mice uniform in 6 of the 7 bacterial groups that were assessed - only *Lactobacillus* remained different between the co-housed WT and NodDKO mice. *Lactobacillus* is known to be an especially adherent strain of bacteria (Styková, Nemcová, Valocký, Novotný, & Guba, 2013), possibly explaining why it was not normalized with our co-housing protocols. Although, the co-housed microbiota structure of both WT and NodDKO mice was uniform (with the exception of *Lactobacillus*), the microbiota profile remained orthogonal to that of both the conventionally housed WT and NodDKO

microbiotas. Upon co-housing, Enterobacteriaceae and SFB were normalized to the lower levels of conventionally housed WT microbiota levels, while *Bacillus*, was normalized to the elevated levels found in conventionally housed NodDKO mice. Probiotic studies in NodDKO mice have also demonstrated that administration in mice does not improve the colonic permeability defects associated with NodDKO mice nor the pro-inflammatory cytokine profile – evidence that the genetic defect directly regulates gut function. (Natividad et al., 2012) However, the mechanism by which Nod-like receptors modulate the microbiota community structure remains to be elucidated. While no bacterial profile has been deemed the gold standard of health, the balance in diversity and ratios among bacterial strains is likely necessary for generalized health. (Koboziev et al., 2014)

To investigate the potential influence of the microbiota on stress-induced behavioral and cognitive deficits resulting from the absence of Nod-like receptors, behavioral studies were performed with NodDKO and WT mice that had been co-housed at weaning and have a similar microbiota community population structure. Contrary to our hypothesis, stress-induced anxiety-like behavior in NodDKO mice was not normalized upon co-housing, indicating that the dysbiosis experienced by NodDKO mice was likely not responsible for the observed behavioral deficits. Thus co-housing NodDKO mice with WT mice did not ameliorate the stress-induced behavioral defects in NodDKO mice as would be expected if dysbiosis was the underlying cause of the anxiety-like behavior. However, the possibility that the

distinct *Lactobacillus* populations in WT and NodDKO mice are also influencing the behavioral deficits cannot be excluded.

With respect to recognition memory, as determined via the NOR test, we found that the stress-induced memory defects observed in conventionally housed NodDKO mice were retained with co-housing. Interestingly, these stress-induced anxiety-like behaviors were actually transferred to the co-housed WT mice, with co-housed WT mice demonstrating a similar level of memory impairment as the conventionally and co-housed NodDKO mice. While unexpected, further studies are required to investigate the nature of this memory defect transfer. Social factors of co-housed mice or the partial normalization of the microbiota serve as potential avenues for further research

An increasing number of studies are seeking to determine a connection between bacterial species and host neural or humoral pathways. GABA, the main inhibitory neurotransmitter of the central nervous system, is important in the development of stress-related psychiatric conditions. (Bravo et al., 2011) Recent studies have demonstrated that long-term administration of some *Lactobacillus* strains can lead to alterations in GABA mRNA expression in the brain (cortical regions, hippocampus and amygdala) as compared to control-fed mice – an effect not observed in vagotomized mice. (Bravo et al., 2011) Furthermore, specific strains of *Lactobacillus* (*L. rhamnosus* JB-1) have been shown to reduce stress-induced corticosterone levels as well as baseline anxiety-like behavior in healthy mice. (Bravo et al., 2011) However, in our study, co-housed NodDKO mice, which

harbored higher proportions of *Lactobacillus* than co-housed WT mice, had elevated anxiety-like behavior in comparison to co-housed WT mice. This suggests Nod1 and Nod2 receptors may be influencing behavior beyond mediating the composition of the microbiota. Assessing GABA levels in the brain of NodDKO mice, both conventionally- and co-housed, will be of great interest in future studies.

The HPA-axis is an important regulator of the host response to a stressful stimulus, with elevated serum corticosterone levels serving as an indicator of activation of the HPA-axis. (Ait-Belgnaoui et al., 2014) Exposure to psychological stress (WAS), for example, induces a robust, and reproducible activation of the HPA-axis. (Gareau et al., 2011) Conventionally housed WT and NodDKO mice responded to exposure to WAS with elevated serum corticosterone levels. However, upon co-housing, the stress-induced HPA-axis activation was reduced and all co-housed animals had a trend towards elevated corticosterone levels in both stressed and unstressed conditions compared to conventionally housed WT mice. Therefore, we can conclude that co-housing can modulate the HPA-axis and can prevent stress-induced HPA-axis activation. The possibility that elevated *Lactobacillus* levels of the co-housed NodDKO and WT mice could explain the reduced HPA-axis activation has not yet been eliminated. (Bravo et al., 2011) However, it is also possible that Nod-like receptors are involved in mediating this altered HPA-axis response.

Future Directions

We have demonstrated in our current studies that Nod-like receptors play an important role in modulating the MGB-axis in mice. Despite these studies, we currently do not know the detailed mechanism by which this is taking place, nor which cell types – immune, gut or brain cells – that express NLRs act in the MGB axis. In future studies, we plan to utilize conditional knockout mice – tissue-specific deletions of Nod receptors on epithelial cells, immune cells and in discrete brain regions involved in mood and cognition – which may be useful in further characterizing the role of Nod1 and Nod2 receptors in the MGB-axis. Although preliminary studies demonstrated no behavioral differences in individually knocking out either Nod1 or Nod2 receptors (Gareau, MG - data not shown), tissue-specific double knockouts could provide an opportunity to understand the precise mechanism by which Nod-like receptors are functioning in the bidirectional communication of the MGB-axis.

Additionally, alternative methods of normalizing the microbiota may provide important information for our understanding of the interplay between the microbiota and the Nod1/Nod2 receptors. Co-housing was not fully successful in normalizing the microbiota as *Lactobacillus* was not normalized and the co-housed mice had a different microbiota community structure from both conventionally housed WT and NodDKO mice. Furthermore, social factors inherently at play with co-housing mice are difficult to eliminate. (Kulesskaya, Karpova, Ma, Tian, & Voikar, 2014) For example, in one study, mice that were subjected to daily WAS were co-

housed with unstressed mice. These unstressed co-housed mice developed WAS-induced small bowel inflammation (enteritis) associated with increased elevated corticosterone, despite never being directly subjected to WAS. (Sun et al., 2013). Furthermore, another group established that depression is contagious between rodents housed together. (Boyko et al., 2015) One possibility for future improved microbiota normalization is fecal microbiota transplant (FMT). Currently a treatment for *Clostridium difficile*, FMT is thought to be effective by expanding the diversity of the recipient patient's microbiome to match that of the healthy donor. (Shahinas et al., 2012) Finally, metagenomics analysis using Illumina sequencing of the microbiota could improve the investigative analysis of the microbiota performed in these studies, allowing for identification of untargeted species of bacteria and those of very low abundance not picked up by qPCR methods.

In conclusion, we demonstrated that Nod1 and Nod2 receptors act as important mediators in the MGB-axis, possibly via the HPA-axis. We found that Nod1 and Nod2 receptors play an important role in mediating memory and anxiety-like behavior following exposure to acute psychological stress. Co-housing mice at weaning normalized the gut microbiota in 6 of 7 bacterial groups studied, while having no effect on stress-induced behavioral defects seen in NodDKO mice. Co-housing also led to the transfer of NodDKO stress-induced recognition memory deficits to WT mice, while limiting the stress-induced HPA-axis activation. Elucidating the mechanism by which Nod-like receptors are linked with cognition,

anxiety and stress may provide the key to treating the behavioral comorbidities in patients suffering from chronic intestinal diseases.

I would like to acknowledge Dr. Kim Barrett, Dr. Melanie Gareau, and Nancy Zhang for their contributions to this project.

V.

APPENDIX: ILLUSTRATIONS and TABLES

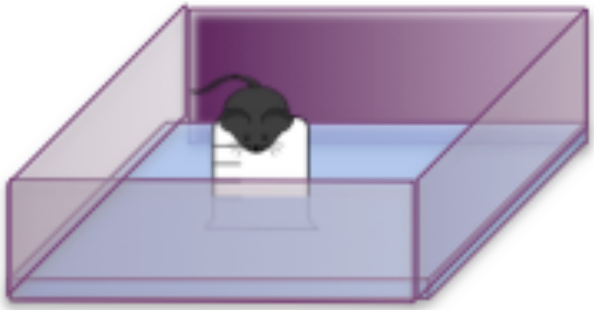


Illustration 1: ***Water Avoidance Stress (WAS) Setup***
Mice are placed on a platform (inverted 50ml beaker) in a cage filled with approximately 2 cm of room temperature water for 1 hour.

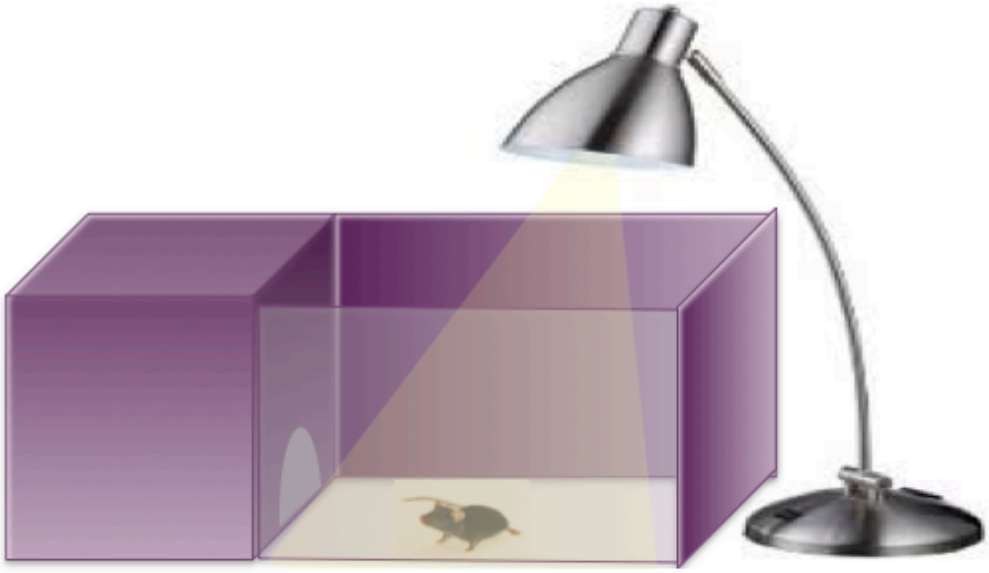


Illustration 2: ***Light/Dark Box Test Apparatus***
Mice are placed in the lit portion of the light/dark box and are allowed to explore the lit and darkened portions of the cage for a total of 10 minutes, which is recorded. The total time the mouse spends in the light is calculated, as well as the frequency of transitions between the light and dark portions of the box.

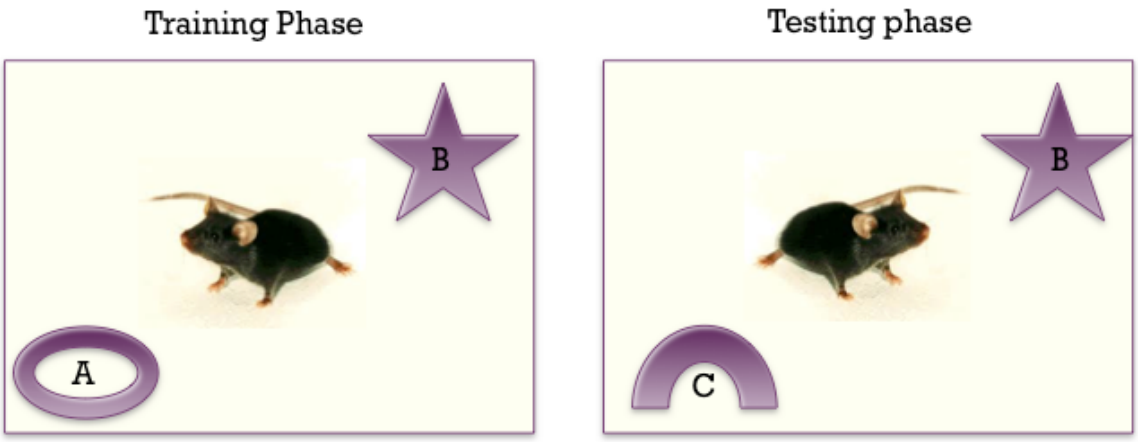


Illustration 3: ***Novel Object Recognition (NOR) Test***
Mice are habituated for 1h in the same cage used for the experiment. During the **training** phase, two novel objects (A and B) are placed in the cage. The mouse is allowed to explore these objects for 5 minutes and behavior is recorded. The objects are then removed from the cage and the mouse rests without any objects in the cage for 15 minutes. Following this rest period, object B from the training phase along with novel object C are placed in the cage during a **testing** phase. The mouse again explores the objects for 5 minutes. The number of times the mouse investigates each object is recorded.

Table 1: ***Primer sequences employed in qPCR analysis of the microbiota***
(Gareau et al., 2011)

Target	Forward (5'-3')	Reverse (5'-3')
Eubacteria	ACTCCTACGGGAGGCAGCAGT	ATTACCGCGGCTGCTGGC
E. Rectale	ACTCCTACGGGAGGCAGC	GCTTCTTAGTCAGGTACCGTCAT
SFB	GACGCTGAGGCATGAGAGCAT	GACGGCACGGATTGTTATTCA
Bacillus	GCGGCGTGCCTAATACATGC	CTTCATCACTCACGCGGCGT
Lactobacillus	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG
Enterobacteriaceae	GTGCCAGCMGCCGCGGTAA	GCCTCAAGGGCACAACCTCCAAG
Bacteroides	GAGAGGAAGGTCCCCCAC	CGCTACTTGGCTGGTTCAG
Firmicutes	GCTGCTAATACCGCATGATATGTC	CAGACGCGAGTCCATCTCAGA

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