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Neuroinflammation, Peripheral Inflammation and Gut Microbiome Profiles in Male Mice from Two Proposed Mouse Models of Social Behavior Deficits

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A thesis

presented to

the faculty of the Department of Biological Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Biological Sciences, Microbiology

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by

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August 2021

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Dr. W. Andrew Clark

Keywords: autism, microbiome, cytokine, IBA-1

ABSTRACT

Neuroinflammation, Peripheral Inflammation and Gut Microbiome Profiles in Male Mice from Two Proposed Mouse Models of Social Behavior Deficits

by

Sarah Parkinson

Autism spectrum disorder (ASD) is a neurodevelopmental disorder marked by social deficits and repetitive actions. A communication pathway exists between the brain and gut called the gut-brain axis. It is thought that gut bacteria can secrete signaling molecules, triggering inflammation across the body. These studies attempt to determine if markers are expressed in two mouse models of ASD behaviors, BTBR and a valproic acid model. Immunohistochemistry of ionized calcium binding adaptor molecule 1 from male mouse brain tissue showed no microglial activation in any group. Cytokine analysis did exhibit an increase in interleukin 1 (IL-1β) in male adult mice only. Sequencing of bacterial profiles demonstrated differences between groups. Altogether, it appears that microbiome differences do not trigger robust differences in inflammatory pathways in these animals in this study. It is imperative that a reliable animal model of behaviors be identified for novel studies that can impact the development of the disorder.

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CHAPTER 1. INTRODUCTION

Autism Spectrum Disorder

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder that was first described by Leo Kanner in 1943. ASD is typically marked by repetitive actions, social deficits, and obsessive behaviors. It is referred to as a spectrum disorder because it encompasses deficits in communication and social behaviors with varying levels of functionality (Srikantha and Mohajeri 2019). For example, some individuals with ASD are unable to communicate at all while others can express themselves effectively (Courchesne et al., 2019). The disorder is usually diagnosed by two years of age and is more likely to affect males rather than females, at a ratio of 5:1 (Rosenfeld 2015, Eshraghi et al. 2018). Furthermore, it currently affects one out of every fifty-four children, with as high as 1 in 42 males (Maenner et al. 2016). Behavioral issues including social and communicative deficits are used to diagnose ASD. Additionally, individuals with ASD can also suffer from a wide range of physiological problems including gastrointestinal issues (Li and Zhou 2016). Also, sleep issues can also be present in ASD. The heterogeneity of symptom patterns in ASD can make it difficult to characterize hallmark ASD behavior symptoms from a comorbidity-related behavior side effects i.e. gut pain or sleeplessness could be a major contributor to the behavior of some ASD individuals (Jyonouchi et al. 2017).

Since its initial characterization in the 1940s, the disorder has increased in prevalence in the United States. However, it is unclear whether the disorder's familiarity has resulted from awareness in diagnosing criteria or if the increase is due to other factors like genetics, epigenetics, or environment (Rosenfeld 2015). Currently, there is no known cause of ASD; however, it is hypothesized that both genetic and environmental factors play a role. It has been

demonstrated in recent literature that maternal immune activation and exposure to certain drugs are associated with an offspring ASD diagnosis (Bristot Silvestrin et al. 2013). There is currently no treatment for ASD; various symptoms like gastrointestinal and developmental issues can be managed through different forms of therapy and medicines. The elusive etiology of ASD has certainly contributed to the lack of drug therapies to treat the core features of the disorder (Hsiao et al. 2013). Overall, a sizable knowledge gap is present in the neurobiological underpinnings of ASD. "ASD living biology" is a term coined to investigate both prenatal and postnatal information in order to gain a better understanding of ASD and its genetic and potential environmental components (Courchesne et al. 2019). Various aspects of ASD living biology have been researched for this particular study in hopes of narrowing down a sufficient animal model to further study the disorder. Biological factors including neuropathology, inflammation, and the gut-brain axis are all potential aspects that could play a role in ASD etiology.

Neuropathology of ASD

Neuropathological changes have been demonstrated in human ASD. It has been shown in multiple studies that males with ASD have anywhere from 36-67% more neurons in the prefrontal cortex than typically developing (TD) males (Courchesne et al. 2019) Because proliferation and differentiation occur during prenatal development, it is hypothesized that a disruption in these processes results in this overabundance of prefrontal neurons. While these data are based on extremely low study numbers, they are thought to contribute to the overall increased brain volumes that have been shown in ASD when compared using postmortem tissues (Courchesne et al. 2019).

Brain region differences have been noted in ASD. Specific brain regions such as the hippocampus have demonstrated differences when comparing ASD to TD human brain tissue. The hippocampus is an elongated structure that can be located in the temporal lobe and is involved with memory formation, processing, and recollection (Knierim 2015). The different subgroups of the hippocampus and the entorhinal cortex form a communication loop—cells in the entorhinal cortex send an input to the dentate gyrus. This input then travels from the dentate gyrus to cortical area 3 (CA3), from CA3 to CA1, and then back to the entorhinal cortex (Knierim 2015, Zeidman and Maguire 2016). In ASD, the CA1 contains an elevated number of pyramidal neurons and an increase in GABA-ergic neurons compared to other areas and in non-ASD individuals (Knierim 2015, Zeidman and Maguire 2016). Another observation in ASD individuals is enlarged axon terminals in both the hippocampus and entorhinal cortex (Varghese et al. 2017). Hippocampal abnormalities have been observed in some cases of ASD, namely the previously mentioned densely populated number of neurons in CA1 to CA4, and less complex dendritic trees and branches in the same areas (Nicholson et al. 2006). In a recent study by Matta et al., their team demonstrated that density of microglia in the dentate gyrus of the hippocampus was elevated in the Neuroligin-3 ($NL3^{R451C}$) model that was used (Matta et al. 2020).

Neuroinflammation in ASD

Neuroinflammation is a key marker that is associated with ASD. Altered cytokine levels and chemokine production in the brain has been shown in ASD previously by our laboratory (Sciara et al. 2020). Additional studies on postmortem brain tissue of ASD individuals reveals increased glial cell activation and an elevated level of proinflammatory cytokines like IL-1β and IL-6, analyzed and supported through immunohistochemistry and ELISA (Pettrelli et al. 2016). It is thought these aberrant cytokine levels are the result of aberrant microglia activation.

Microglia are the immune cells of the central nervous system. When microglia perform phagocytosis in the brain that includes synaptic remodeling and pruning of unnecessary cells, they are referred to as activated (Koyama and Ikegaya 2015). Activated microglia have a morphologically distinct shape and they release cytokines (Pettrelli et al. 2020) Inflammation in the brain arises from premature stimulation and proliferation of microglia (Matta et al. 2019). Prematurely activated microglia can interrupt pruning that is thought to lead to developmental issues (Kim et al. 2018). Interruptions in pruning has been shown to result in learning disabilities and social behavior deficits (Petrelli et al. 2016). It has been demonstrated in a study by Suzuki et al. that evaluated twenty ASD and twenty normal males that the ASD patients typically have excessive microglia activation in the corpus callosum, anterior cingulate cortex, and cerebellum compared to non-ASD patients, as visualized through PET and MRI scanning (Suzuki et al. 2013). The microglia-specific protein, ionized calcium binding adaptor molecule 1 (IBA-1), is a marker for microglia due to its activity in actin bundling/rearrangement and membrane ruffling (Ahmed et al. 2007). Immunohistochemistry using IBA-1 has implicated microglia activation, but not increased numbers, in the dorsolateral prefrontal, fusiform and visual cortices in small sample sizes of human pathological samples (Liao et al. 2020). As substances cross into the brain, microglia would be the first line of defense.

The blood-brain barrier (BBB) is a fundamental component of the nervous system. The BBB functions to keep harmful cells and molecules out of the brain. A faulty barrier would allow a plethora of immune cells, chemokines, cytokines, and other complex molecules to enter the brain (Fiorentino et al. 2016). It has been suggested that the increase in density of activated microglia and presence of certain cytokines is due to extraneous molecules crossing the BBB.

This hypothesis is further supported through the research of animal models where activated microglia are associated with a deficient blood-brain barrier (Da Fonseca et al. 2014).

Peripheral Cytokines

Cytokines are pleiotropic signaling proteins that are produced by different types of activated immune cells throughout the body including the brain (as mentioned above) to serve as the primary element of an inflammatory response (Mousa et al. 2013). Cytokines play an integral role in both innate and adaptive immunity. There are two primary types of cytokines, proinflammatory and anti-inflammatory. Proinflammatory cytokines elicit inflammatory response. Anti-inflammatory cytokines can regulate the proinflammatory response, thus suppressing inflammation, and, in some instances, even pain (Zhang and An 2007). Varying amounts of pro-inflammatory and anti-inflammatory cytokines have been found in brain, blood and cerebrospinal fluid in ASD patients (Masi et al. 2015). Cytokine imbalances, aberrant control of T-cells, or premature activation of microglial cells in the brain can cause conformational changes leading to deficits concurrent with those found in ASD (Eshraghi et al. 2018).

Interleukin 1, subunit beta (IL-1β) is a proinflammatory cytokine that impacts the development of neurological cells and processes in the brain. It has been associated with synapse regulation in the hippocampus (Ashwood et al. 2011). IL-1β is produced by microglia as a response to stimuli (Ashwood et al. 2011). Examples of stimulation that can lead to IL-1β production and secretion are events such as injury to cells and inflammation (Zhang and An, 2007). Aberrant increased levels of IL-1β have been linked to memory, social, and learning deficits (Goines et al. 2012). Microglia upregulate production of IL-1β through neurotensin

stimulation and both IL-1β and neurotensin have demonstrated increases in ASD individuals when compared to neurotypical individuals (Tsilioni and Theoharides 2018). Serum from sixteen male and four female ASD patients revealed that extracellular vesicles (exosomes) present in serum were associated with increased IL-1β stimulation and secretion in ASD patients when compare to TD control patients (Tsilioni and Theoharides 2018). Other studies, such as the Kordulewska study from 2019, also revealed an increased IL-1β concentration in ASD subjects compared to controls. It is important to note that this study used human ASD subjects instead of mouse models but exhibited increased levels in fetal brains (Kordulewska et al. 2019)

Interleukin 6, IL-6, is a proinflammatory cytokine that can impact brain development. IL-6 is able to cross the blood-brain-barrier and has been shown to impact fever and body temperature (Kordulewska et al. 2019). IL-6 is also involved in both astrocyte and microglial activation in the brain (Zhang and An 2007). It has been shown that IL-6, along with other proinflammatory cytokines including tumor necrosis factor alpha (TNF-α) and IL-1β, have been found in higher concentrations in serum from ASD children compared to neurotypical children (Prosperi et al. 2019). Behavior, GABA dysregulation, and a number of other processes have been implicated in individuals with higher concentrations of IL-6 (Goines et al. 2012). Altering IL-6 levels with anti-inflammatory substances has been shown to reduce obsessive behaviors in animal models (Matta et al. 2019). Additional support was shown by Sauer et al. where anti-IL-6 antibodies reduced some obsessive ASD-like behavior deficits (Sauer et al. 2019). (This study doesn't mention specific behavior, just that behavior was improved after treatment with these antibodies) Treatment with anti-IL-6 therapeutics and the subsequent improvement of deficiencies in animals does indicate that IL-6 could be an important cytokine in the pathophysiology of the disorder (Wei et al. 2013).

Tumor necrosis factor alpha, TNF-α, is another proinflammatory cytokine that has been found to be upregulated in ASD individuals (Prosperi et al. 2019). It is known that TNF-α plays a role in regulation of homeostasis, synaptic plasticity, and cell signaling to activate other downstream factors and pathways in the brain (Muhammad 2019). The activation of nuclear factor kappa B (NF-κB) occur through a signal cascade that begins with TNF-α binding to TNF receptors, TNFR1 and TNFR2. TNF- binding recruits additional molecules including those related to the MAPkinase pathway. Ultimately, phosphorylation, ubiquitination, and degradation of TNF-α binding leads to the activation of the highly conserved NF-κB transcription factor (Albensi 2019). Because of TNF alpha binding, NF-κB is thus upregulated in ASD. These findings suggest that TNF-α may be an important marker of neuroinflammation in ASD (Young et al. 2011, Muhammad 2019).

In contrast, anti-inflammatory cytokines can aid in the regulation of the aforementioned proinflammatory cytokines. For instance, IL-10 is known to subdue proinflammatory cytokines like TNF-α and IL-6 (Zhang and An 2007). Additionally, it was observed that different IL-1β/IL-10 ratios were found in forty percent of ASD patients and were associated with increased IL-6 levels in those patients (Jyonouchi et al. 2019). Also, the same group had previously found a correlation between microRNA (miRNA) expression, behavior, and IL-1β/IL-10 ratios (Jyonouchi et al. 2017).

Microbiome in ASD

The gut microbiome is a critical component of a healthy individual. The gut microbiome is collectively all the micro-organisms that live in the alimentary or digestive tract. The digestive tract contains varying numbers of microorganisms in a human. A few micro-organisms are

found in the stomach, but concentration gradually increases through the small intestine and into the colon where the most diverse micro-organisms can be found in the human body (Mangiola et al. 2016). There are approximately 10^{13} microorganisms including bacteria, viruses, and yeasts that can be found in the human gut (Bonaz et al. 2018). Microorganisms in the gut participate in various functions in the body including molecular digestion, furthering the maturation and differentiation of the immune system, and creating an intestinal barrier to protect from harmful pathogens (Mangiola 2016). Regarding digestion, gut bacteria aid in the synthesis of various vitamins and nutrients as well as metabolize complex molecules like carbohydrates, sugars, and lipids (Rosenfeld 2015). Metabolism of these molecules would be extremely difficult or even impossible for the body if not for the aid of gut microbiota.

Bacterial colonization of the gut happens at birth as infants passing through the birth canal are exposed to a variety of microbes and are influenced by breastmilk. Interestingly, infants that are not breastfed or that are born via Caesarean section still develop a bacterial profile due to incompletely characterized bacterial exposures (Rosenfeld 2015). A child's gut flora will match that of the mother for the first several months of life but quickly becomes unique to the individual—even monozygotic twins do not have the same gut microbiome (Rosenfeld 2015). However, dysbiosis of the gut flora have been associated with a variety of medical conditions including obesity, autoimmune disorders, Alzheimer's, and autism spectrum disorder (Mangiola et al. 2016). It has been observed that individuals with ASD have differences in gut microbiota and increased immune response compared to those without ASD (Eshraghi et al. 2018). Though individuals with ASD have altered gut flora, it is unclear whether this is due to co-morbid gastrointestinal issues or if ASD causes the dysbiosis (Vuong and Hsiao 2017).

Specific phylogenetic differences have been identified in some but not all ASD patients. It has been shown that children with ASD have an imbalance in the Bacteroidetes/Firmicutes ratio when compared to neurotypical children that exhibited reduced amounts of Bacteroidetes, but higher amounts of Firmicutes and Proteobacteria (Luna et al. 2016). Interestingly, the most populous phyla of bacteria in the digestive tract are Bacteroides and Firmicutes (Mangiola 2016). Additionally, Bacteroides and Firmicutes are the most abundant phyla in the mouse gut with Sauer et al. also finding an imbalance of these phyla in their ASD mouse model, a SHANK3 knockout, when compared to the control model (Sauer et al. 2019). It has been shown that the most abundant bacteria is the genus Clostridium in the gut of ASD individuals when compared to TD control individuals (Mangiola 2016, Sauer et al. 2019). Because an offspring's microbiota will match the mother's briefly after birth, it is reasonable to assume that any dysbiosis present in the mother's gut will be inherited by the child and could increase risk of neurodevelopmental issues, potentially from factors like maternal immune activation (Kim et al. 2017).

Overall, the alteration of bacterial phyla expression in the gut has gained significant attention in recent years in neurodevelopmental disorders. Segmented filamentous bacteria (SFB) of the Firmicutes phyla were shown to alter ASD-like symptoms in mice treated with poly I:C to stimulate maternal immune activation (Kim et al. 2017). In the same study, vancomycin-treated poly I:C injected dams produced offspring that demonstrated little to no behavioral or social deficits while dams that were not treated with vancomycin had offspring that exhibited repetitive behavior, social delays, and developmental delays (Kim et al. 2017). Additionally, the study also revealed that a higher colonization of SFB in the gut of pregnant dams was associated with an increase in the amount of Th17 cells present and increased concentrations of IL-17. The strain with SFB displayed behavioral deficits concurrent with those of ASD, while the other strain

lacking SFB did not display behavioral abnormalities or an increase of IL-17a in blood serum (Kim et al. 2017). It has been shown that SFB concentration is directly correlated to the number of Th17 cells and IL-17a levels in blood (Kim et al. 2017). Based on these data, it cannot be confirmed that the presence of SFB is the single causative agent of social behavior deficits, but it may be a key factor that requires further investigation to the relationship to ASD-like symptoms (Kim et al. 2017). Additionally, it has been shown in mice that administration of Bacteroides fragilis to the gut actually helped to alleviate gaps in the intestinal cell barrier and the administration of other Bacteroides species was shown to reduce ASD-like symptoms and behavior in children after treatment (Hsiao et al. 2013, Evrensel and Ceylan 2016). It is interesting that the alteration of gut species can directly affect social behaviors.

Gut-Brain Axis

The gut microbiome has been found to communicate with other organ systems, as in the case of the gut-brain axis. A bidirectional pathway consists of direct or indirect by-products produced by the gut bacteria that can signal to cells in the brain and alternatively neurotransmitter release and the vagus nerve pathway can stimulate the gut. The gut-brain axis is thought to be the primary link between the CNS, bacterial species in the gut, and the GI tract (Luna et al. 2016).

Communication along the gut-brain axis can occur through many pathways. However, the vagus nerve (VN) is a vital component in the gut-brain axis. This nerve, which travels from the brainstem to the large intestine, is the longest nerve in the body and senses inflammatory molecules, promotes peristalsis, and is a modulator of homeostasis in the body (Bonaz et al. 2016). As part of the autonomic nervous system, the vagus nerve serves as a mediator between the gut and the central nervous system. It is able to directly and indirectly sense microorganisms

and their metabolites in the gut via afferent fibers to pass this information to the brain to elicit a peripheral body for a response (Bonaz et al. 2018). Both afferent and efferent fibers comprise the vagus nerve. Afferent and efferent fibers differ in myelination, diameter, function, and amount of fibers present—the vast majority of fibers are afferent (Beaumont et al. 2017). These fibers interact with intestinal epithelial cells, express TLR-4, and can specifically sense proinflammatory cytokines. All of these mechanisms allow the vagus nerve to mediate signals to the brain (Bonaz et al. 2016).

In 2005, the U.S. Food and Drug Administration approved vagus nerve stimulation (VNS) as a form of alternative treatment for depression and epilepsy. The vagus nerve can be stimulated in a variety of ways including an invasive procedure that involves a surgically implanted generator that sends a series of impulses to an electrode that surrounds the left cervical vagus nerve (Jin and Kong 2017). However, there are also noninvasive approaches to stimulate the vagus nerve such as massage therapy and auricular stimulation (Engineer, Hays, and Kilgard 2017). VNS has not been approved as a form of treatment for ASD or other neurodevelopmental disorders but has shown promising results (Jin and Kong 2017). Low vagal activity is associated with speech delays, a lack in expression, and auditory processing. After VNS, a higher amount of vagal activity appears to have a positive effect on mood and behavior (Jin and Kong 2017). Additionally, language impairment, memory, and aspects of auditory processing were also positively affected by VNS in children with autism (Engineer, Hays, and Kilgard 2017). Afferent vagal fiber activation can also alter serotonin, GABA and glutamate levels in the brain, and animals administered with *Lactobacilli* demonstrated a reduction in stress and depressive behavior (Bonaz et al. 2018). It is possible that a combination therapy, such as VNS and probiotic administration, could be a potential treatment regimen for ASD in the future.

In recent studies, it has been shown that short-chain fatty acids, by-products of various bacterial species, could also be linked to ASD. Certain microorganisms, such as Clostridium, a species that has been shown to exist in higher levels in ASD individuals, are unable to catabolize certain SCFAs—resulting in these fermentation products being isolated in high amounts (Mahajan et al. 2019). Increased amounts of the SCFA, propionic acid, have been shown to induce motor function abnormalities and have elevated neuroinflammation (MacFabe et al. 2012). Additionally, patients with ASD exhibit increased levels of propionic acid in human waste products (Mahajan et al. 2019). It is thought that immune dysregulation that is associated with SCFA production may occur throughout the body (Rosenfeld 2015). *Lactobacillus helveticus*, a member of the Firmicute phyla, occupies a significant role in host immune responses and in fecal profiles (Taverniti and Guglielmetti 2012). The *Lactobacillus* species aid in optimizing the vaginal mucosa by controlling *Candida* yeast overgrowth in the vaginal tract; thus, contributing to a healthy microbiota of offspring passing through the vaginal canal during the birthing process (Taverniti and Guglielmetti 2012). Additionally, *Lactobacillus helveticus* aids in the conversion of lactate to butyrate, a beneficial SFCA that is essential in gut maintenance and cell growth. It has been shown that mice administered milk fermented with strains of *Lactobacillus helveticus* express a lower concentration of the proinflammatory cytokine IL-6 compared to mice that did not receive the *Lactobacillus helveticus*-fermented milk (Taverniti and Guglielmetti 2012).

The gut microbiota is a wildly intriguing community due to the numerous downstream influences. For instance, the bacterial species present in the gut microbiota can elicit signals that can be transmitted through the blood to other areas of the body, like the liver (Scarpellini et al. 2020). It is possible that dysbiosis of the gut can lead to aberrant inflammatory cytokine

production. Additionally, it is tempting to speculate that tight junctions between GI epithelial cells can trigger inflammation in other areas of the body i.e. the liver (Scarpellini et al. 2020).

The intestines have a protective barrier. This intestinal barrier is made up of three primary components—1) tightly linked epithelial cells, 2) a layer of mucous, and 3) multiple microorganisms. In typically developing children without intestinal or immune-related disorders, this barrier will remain intact. It has been postulated in ASD that the intestinal barrier has been compromised leading to a higher incidence of gastrointestinal issues in children with ASD (Mahajan et al. 2019). It is thought that the tight junctions between epithelial cells becoming weakened due to changes in mucosal thickness thus affecting overall permeability (Samsam et al. 2014). Substances that are produced by bacteria can enter through the compromised space in the intestine to travel to the periphery or possibly directly penetrate the blood-brain barrier inciting an immune response that leads to activated glial cells (Samsam et al. 2014). Therefore, it is important that the development and maintenance of the intestinal barrier stay unaffected and protected from outside influences that can harm and alter the gut microbiota. Diet is the most direct way to influence the gut microbiome, but in severe cases of GI issues, the microbiota can even be replaced (Aroniadis and Brandt 2013). Some individuals with gastrointestinal issues have gone through a process called fecal microbiota transplantation (FMT) (Aroniadis and Brandt 2013). First performed in the fourth century, this ancient technique was forgotten for hundreds of years but resurfaced as a form of treatment for bacteria imbalance in the gut in the twentieth century and can now be performed via a donor or a sample culture (Evrensel and Ceylan 2016). FMT is a widely accepted treatment with few side effects for people with *Clostridium difficile* infections (Aroniadis and Brandt 2013). Interestingly, the *Clostridium* species is more prevalent in the feces of ASD individuals compared to healthy individuals

(Aroniadis and Brandt 2013). In the few FMT studies that have been performed, symptoms were less severe after successful transplantation. Though positive results have been achieved thus far, further research would need to be performed to determine the overall efficacy as an accepted treatment for ASD and other neurodevelopmental and neuropsychiatric disorders (Aroniadis and Brandt 2013, Evrensel and Ceylan 2016).

It is tempting to speculate that the microbiota influences serotonin production in ASD. Serotonin, sometimes referred to as 5-hydroxytryptamine (5HT), is a well-researched neurotransmitter that is a derivative of the amino acid tryptophan (Agus et al. 2018). It is generated by tryptophan hydroxylase and has been associated for a number of years with disorders like ASD. An association has been observed in multiple studies between elevated plasma serotonin levels and patients with ASD-like symptoms (Guo and Commons 2017, Tanaka et al. 2018). Serotonin is most commonly associated with the brain, but it is predominately produced in the gut via intestinal cells. In the gut, 5HT plays a role in signaling to both intrinsic and extrinsic neurons, peristalsis, and various other intestinal functions (Agus et al. 2018).

Mouse Models

An important need in the ASD literature is validation of the current animal models that are being used for experimentation. The BTBR strain is a genetically inbred model that has a reduced corpus callosum (Coretti et al. 2017). A size reduction of the corpus callosum has been shown in human ASD pathology and MRI studies when compared to neurotypical individual (McTighe et al. 2013). Additionally, BTBR mice display repetitive grooming behavior and decreased sociability around other mice (McTighe et al. 2013). Neuropathology of brain development shows the BTBR model is altered when compared to the wild type C57BL/6J strain including hippocampal abnormalities, serotonin abnormalities and changes in the expression of

brain-derived neurotrophic factor (BDNF) (Coretti et al. 2017). BDNF and serotonin have a dually regulating relationship where BDNF serves to facilitate serotonin axons and serotonin regulates BDNF expression (Guo and Commons 2017). Common neurobiological hallmarks of the BTBR model are the thin corpus callosum mentioned above and reduced hippocampal neurogenesis. It is also interesting to note that white matter has been found in the cingulate cortex but reduced white matter has been reported in the frontal lobe of this strain (Chadman et al. 2012). Both of these findings have been demonstrated in ASD patients (Chadman et al. 2012).

Lastly, the valproic acid model also uses intraperitoneal administration of valproic acid to C57BL/6J mice during gestation. Valproic acid is a short-chain fatty acid found naturally in the gut and is commonly used as an anti-seizure medication. Epidemiology studies in ASD have shown an increase in ASD in children where mothers have been exposed to valproic acid during the pregnancy; for this reason, a drug safety alert was placed on valproic acid to bring awareness about of potential defects and complications to offspring (Roullet et al. 2013). Animal studies have shown that valproic acid administration to pregnant dams results in microglial activation in the hippocampus including increased size and density of these cells in the offspring (Matta et al., 2019). The exact etiology of valproic acid's effects is unknown, but it is postulated that valproic acid can interfere with biological processes like histone acetylation and DNA methylation, along with increasing gamma-aminobutyric acid concentrations in the brain (Chomiak et al. 2013).

The studies here examine if neuroinflammation, peripheral inflammation and gut dysbiosis are present in the BTBR and VA mouse models. The mouse strain C57BL/6J is used as the wild-type control strain throughout ASD literature. These mice demonstrate increased sociability and show a strong genetic resemblance. It is imperative that a mouse model be

identified that exhibits behaviors and pathophysiology found in human ASD if a treatment is to be identified.

Hypothesis

ASD likely has a multifactorial etiology that includes both genetic and environmental exposures. This study is important in attempting to identify specific genetic and/or environmental factors that could be used to study ASD behaviors. It is hypothesized that the chosen animal models may display inflammatory markers including neuroinflammation, cytokine levels, and gut biome changes that can be identified in the mice. In the proposed experiments, two different mouse models used in ASD experimentation in the literature will be evaluated for alterations in microglia activation, fecal, and cytokine profiles when compared the control strain C57BL/6J.

CHAPTER 2. MATERIALS AND METHODS

Animals

The C57BL/6J mouse strain used for this series of experiments was obtained from Jackson Laboratories (strain #000664, Bar Harbor, Maine). Mice were housed in the Animal Facility at East Tennessee State University. All mice were housed in plastic cages and had unlimited access to food and water. Additionally, all animals, both dams and offspring, received the same daily chow at the ETSU animal facility. Valproic acid (Sigma cat #1069-55-5; St. Louis, Missouri) was administered intraperitoneally to pregnant dams at gestational day eleven. The BTBRT+Itpr3tf/J (BTBR) strain of mice was also obtained from Jackson Laboratories (strain #002282, Bar Harbor, Maine). Breeding was performed for BTBR mice and all offspring were obtained at the ETSU breeding facility. Again, all mice were fed the same daily chow and water ad libitum. Mice were observed for behavior and sacrificed at either postnatal day 21 (P21) known as weanlings or postnatal day 105 (P105) that is considered an adult mouse. Brain tissue and blood were obtained from both weanlings and adults, while fecal samples were obtained from only the adult animals. Both male and female mice were used in all studies. Brain tissues were harvested and fixed in a 4% paraformaldehyde solution for 18 hours at 4^{o}C. Brains were then placed in a 20% sucrose solution for 24 hours at 4^{o}C. Brains were then transferred to a dry vial and stored at -80^{o}C until use. Blood was obtained via a trunk bleed procedure. Feces were obtained daily for 14 days from single animals and stored at -80^{o}C immediately following collection. Animal protocols were approved by the UCAC committee at East Tennessee State University (protocol #171101).

IBA-1Immunohistochemistry

Formaldehyde-fixed, cryopreserved brain tissues were sectioned using a Leica cryostat at 40-micron thickness and stored for less than 24 hours in phosphate buffered saline (.02 mM) at 4^{o}C until immunolabeling. Animals were sectioned in batches. A batch consisted of a control, BTBR, and VA animals. Tissues were handled as a batch throughout the labeling and analysis process. Coronal sections from a batch were first permeabilized in 0.5% bovine serum albumin, 0.4% Triton X-100, and PBS (.02 mM) for twenty minutes. Sections were then blocked for two hours in PBS, 1% BSA, and 0.4% Triton X-100 for two hours. Tissues were incubated for eighteen hours overnight at 4^{o} Celsius with anti-IBA-1 rabbit polyclonal antibody (1:1000; Wako Scientific, Japan). The following day, sections were washed four times in PBS and permeabilized in 0.5% bovine serum albumin (BSA), 0.4% Triton X-100, and PBS for twenty minutes. AlexaFluor488 donkey anti-rabbit IgG (1:200; Thermo Fisher Scientific; Waltham, MA) was diluted in 1% BSA, 0.4% Triton X-100, and PBS and sections incubated in this solution for two hours on a rocking platform in the dark. After incubation, free-floating tissues were washed four times in PBS, mounted on charged glass microscope slides, and coverslipped using Prolong Gold antifade reagent DAPI mounting media (ThermoFisher Scientific; Waltham, MA). Coverslipped slides were stored in slide boxes at 4^{o} Celsius and protected from light until fluorescent imaging.

IBA-1 Imaging and Analysis

Slides were imaged using an EVOS FL auto imaging system at 20X magnification. Every batch (one animal from each of the four groups stained simultaneously) was visualized under the same parameters for DAPI (4',6-Diamidino-2-Phenylindole) and GFP (green fluorescent protein) filters. Light intensity was manually set for all images by using the centimeter slide ruler by setting the instrument slide bar to 2.3 cm. Brightness for DAPI was manually set to 50 and

contrast was set to 40 for all images. GFP brightness was manually set to 55 and contrast to 39 for all images. The overlay image combining the two filters was visualized at a brightness of 50 and a contrast of 43 for all images. A composite or stitched image was taken of each piece of tissue per slide (two positive and one negative piece of tissue) per animal tissue (N=8 males). A DAPI-only, IBA-1 only (GFP) and overlay image were all acquired for every piece of tissue. NIH ImageJ software was used to analyze the intensity of image. A box was placed around the hippocampal hilum, the molecular layer of the hippocampus, and the visual cortex. Fluorescent images were converted to 8-bit files and threshold was adjusted to 5-10% for each piece of tissue per batch of four animals (N=8). Percent area calculations were then generated using Image J and exported to Microsoft Excel and GraphPad Prism 8 for statistical analysis.

Peripheral Cytokine Assay

Serum was placed in a Sarstadt container and separated using centrifugation at 10,000 x g for 5 minutes for P21 males (N=9) and P105 males (N=6). Cytokines (IL-1B, IL-6, IL-10, IL-17a, IFN-gamma and TNF-α levels) were quantified using a Bio-Plex Pro Mouse Cytokine Standard 23-Plex, Group 1 Assay Kit (#M60009RDPD; BioRad; Hercules, CA). Buffers were brought to room temperature until needed. Frozen blood samples were thawed on ice and diluted with sample diluent at a ratio of 1:3 (40 uL blood, 80 uL sample diluent). Wash buffer (1X) was prepared according to manufacturer dilution instruction and applied using a BioRad wash station (BioRad #30034376). The supplied molecular standards were reconstituted in 500 uL of standard diluent, vortexed for five seconds, and then incubated on ice for thirty minutes. A fourfold standard dilution series and blank were prepared according to Bio-Plex guidelines. Magnetic (20X) coupled beads were vortexed for thirty seconds and diluted to 1X in Bio-Plex assay buffer by combining 288 uL of 20X beads and 5,472 uL of assay buffer to yield a total volume of 5,760

uL. Diluted beads were vortexed for fifteen seconds and 50 uL was added to each well of the assay plate. The plate was then washed two times with 100 uL of Bio-Plex wash buffer. Samples, standards, and blank were vortexed and 50 uL of each was added to each well according to pre-planned plate layout. The plate was then covered with sealing tape and left to incubate on a plate shaker at room temperature at 850 rpm for one hour. Detection antibodies (20X) were vortexed for five seconds and centrifuged briefly to collect liquid. The detection antibodies were diluted to 1X by combining 150 uL of detection antibodies and 2,850 uL of detection antibody diluent to yield a total volume of 3,000 uL. The plate was removed from the shaker and then placed in the plate washer to be washed three times with 100 uL wash buffer. Diluted detection antibodies were vortexed and 25 uL was added to each well. The plate was then covered with sealing tape and left to incubate on a plate shaker at 850 rpm for thirty minutes at room temperature. 100X Streptavidin-PE was vortexed for five seconds, centrifuged to collect liquid, and then diluted to 1X by combining 60 uL of streptavidin-PE and 5,940 uL of assay buffer to yield a total volume of 6,000 uL. The plate again was removed from the plate shaker and placed in the plate washer to be washed three times with 100 uL wash buffer. 1x SA-PE (streptavidin-phycoerythrin) was vortexed and 50 uL was then added to each well. The plate was covered again with sealing tape and placed on a plate shaker to incubate at 850 rpm for ten minutes at room temperature. The plate was then washed three more times in the plate washer with 100 uL wash buffer. Beads were resuspended in 125 uL assay buffer, the plate was covered again with sealing tape, and the plate was placed on the plate shaker for thirty seconds at 850 rpm. Finally, sealing tape was removed and samples were exposed to light specific for wavelengths for each fluorophore using a Bio-Plex MAGPIX system.

DNA Isolation

A Qiagen Powersoil DNA isolation kit (Cat no.12888, Qiagen, Germantown, MD) was used to isolate DNA from mouse fecal samples. Fecal matter (250 mg) from each animal was added to a PowerBead tube and then vortexed for one minute. Solution C1 (SDS cell lysis agent) was added and the tube was vortexed briefly. PowerBead tubes were then secured to the vortex via an adapter that would allow for vortexing ten samples at maximum speed for ten minutes. Tubes were centrifuged at 10,000 x g for thirty seconds. The supernatant was then transferred to a clean two mL collection tube followed by the addition of 250 uL of solution C2 (non-DNA organic/inorganic material precipitator reagent) and vortexed for five seconds. Tubes were then incubated at 4°C for five minutes, followed by a one-minute centrifugation at 10,000 x g. After which 600 uL of supernatant was transferred to a clean two mL collection tube where 200 uL of solution C3 (additional non-DNA organic/inorganic material precipitator reagent) was added and tubes were vortexed briefly. The tubes were then incubated on ice for five minutes. Tubes were centrifuged for one minute at 10,000 x g and followed by the addition of 750 uL of supernatant to a clean two mL collection tube. Solution C4 (1200 uL; high-concentration salt solution) was added and vortexed for five seconds. Next, 675 uL of sample was loaded onto a spin column provided in the Qiagen kit, centrifuged at 10,000 x g for one minute, and flow through was discarded. This step was repeated twice or until all of the sample was processed. Solution C5 (500 uL; ethanol-based wash solution) was added to the spin column and centrifuged at 10,000 x g for thirty seconds. Flow through was discarded and the tube was centrifuged again for one minute at the same speed. The spin column was then placed into a clean two mL collection tube where 100 uL of nuclease-free water was added to the center of the white filter membrane and allowed to incubate at room temperature for five minutes. Tubes were centrifuged for thirty

seconds at 10,000 x g to elute DNA. Samples were quantified using a Qubit fluorometric quantification meter. The broad range detection setting was used for DNA quantification.

16S RNA Microbiome Sequencing

Library preparation for DNA sequencing was performed by the Molecular Biology Core Facility at East Tennessee State University. In brief, the 16S ribosomal gene sequence was amplified and indexing sequences for multiplex sequencing were added to DNA amplicons using the Swift Amplicon 16S+ITS Panel (Swift Biosciences, Ann Arbor, MI). This panel facilitates Next Generation Sequencing analysis of complex microbial communities using a single multiplexed primer pool targeting all variable regions of the 16S rRNA gene as well as ITS1 and ITS2 spacer regions. Library sequencing was performed using an Illumina MiSeq sequencer at the University of Tennessee Next-Gen Illumina Sequencing Core Facility (Knoxville, TN).

16S RNA Sequence Analysis

The sequences were imported into the Qiagen CLC Genomics Workbench software where reads were trimmed and aligned with bacterial genomic database for a 97% match. Operational Taxonomic Units were clustered, and species were sorted at the phylum level for analysis. CLC Genomics Workbench software was used to generate a differential abundance analysis graph, heat map, phylogenetic tree, alpha diversity, phylogenetic diversity, beta diversity, and a PERMANOVA was ran to compare all groups and test for significance.

Statistics

All immunohistochemistry and cytokine data were graphed and analyzed using GraphPad Prism V9. Outliers for groups were determined using a ROUT and Grubbs test available with GraphPad. Data for the immunohistochemistry and blood serums were compared using an unpaired Students t-test to compare wild type control to each of the two models. Significance

was determined if the p-value was less than .05. Microbiome data was analyzed, and figures were created using the Qiagen CLC Genomics Workbench software.

CHAPTER 3. RESULTS

Microglia Immunohistochemistry in the Hippocampus

The microglial marker, IBA-1, was used to determine if microglia exhibited altered activation patterns in the mice treated with valproic acid, as well as in the BTBR genetic mouse strain. Immunohistochemistry of IBA-1 in both the hilum and visual cortex of the mouse hippocampus demonstrated no change in area fraction when comparing wild type C57 mice to BTBR or VA mice (**Figure 1 A-C**).

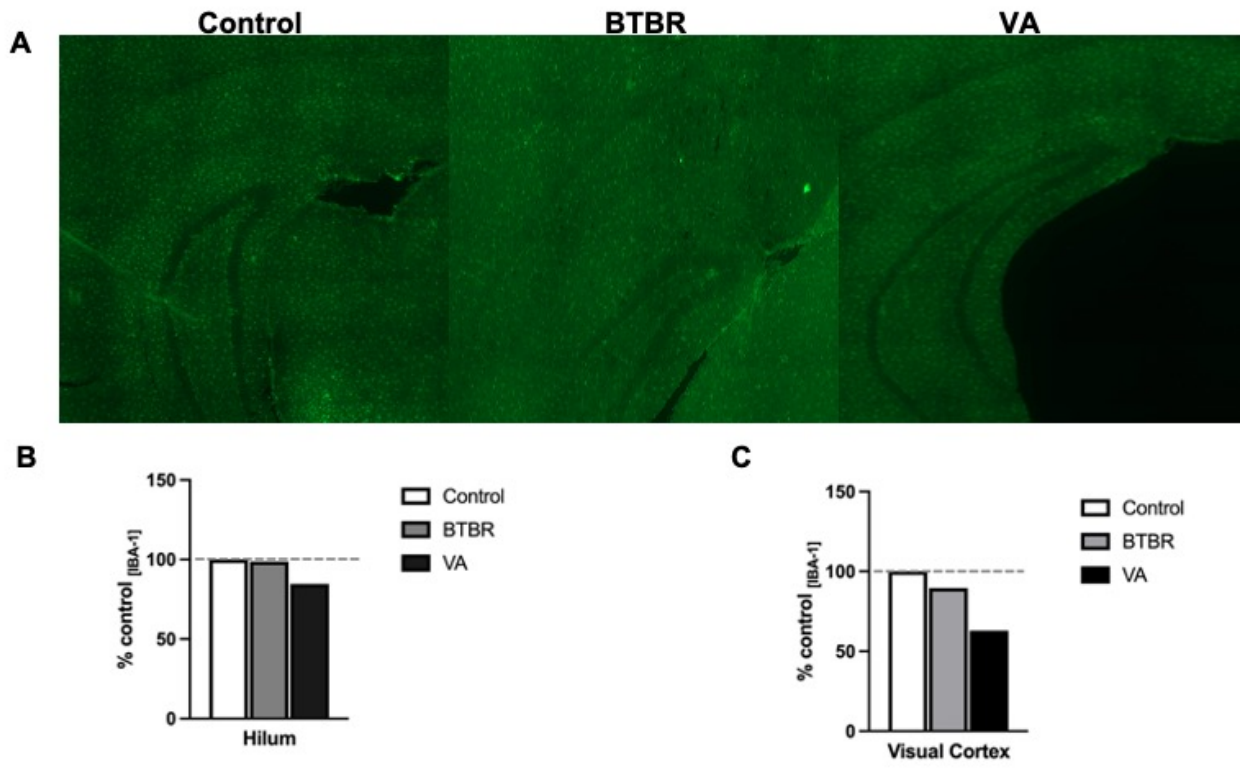

Figure 1. Panel A is representative images of IBA-1 immunohistochemistry at the level of the hippocampus in the mouse brain using proposed mouse models of ASD-like behaviors, BTBR and valproic acid (VA). Panel B is the area fraction shown as percent control of IBA-1 immunoreactivity in the three groups Control (N=8), BTBR (N=8), and VA (N=8) in the hilum. Panel C is the area fraction shown as percent control of IBA-1 immunoreactivity in the three groups control (N=8), BTBR (N=8), and VA (N=8) in the visual cortex. **Percent control of**

area fraction was compared using a Student's t-test between the control and each group. A p-value of less than .05 was used to determine significant differences between groups.

Peripheral Cytokine Levels in Blood Serum

An ELISA assay using blood serum was performed to determine levels of the following cytokines: IL-1β, IL-6, IL-10, IL-17, INF-γ, and TNF-α in the three mouse groups **(Figure 2, Panel A-F).** A list of cytokines used in this assay is displayed in **Table 1.** Two age groups of male mice were analyzed including transitional weanlings at postnatal day 21 (P21) and a fully developed adult group at postnatal day 105 (P105).

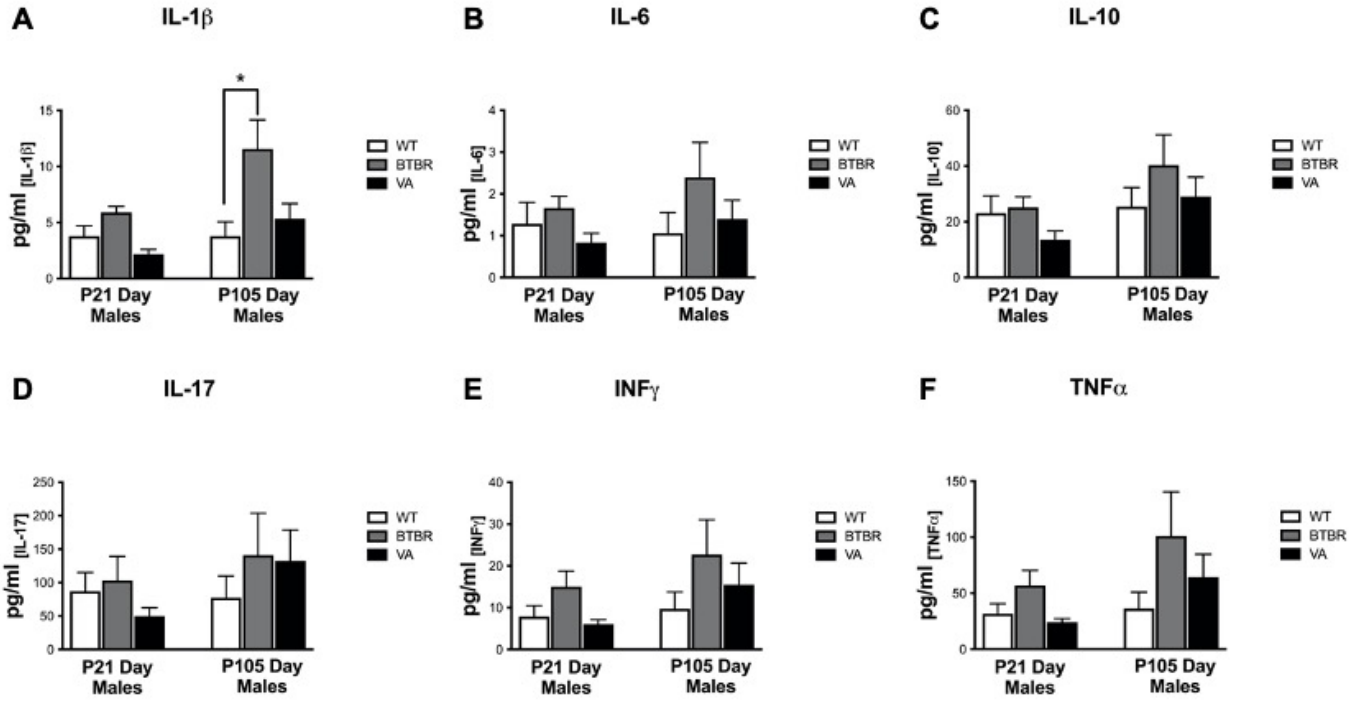

Figure 2. Panel A-F are serum cytokine concentrations were measured on a scale of pg/mL using standard concentrations for each cytokine. White bars indicate wildtype, gray bars indicate BTBR, and black bars indicate valproic acid mice. Two different groups of mice were evaluated for each cytokine including P21 day old weaning mice (N=9 for each group) and P105 adult mice (N=6 for each group). Panel A is interleukin 1, subunit beta (IL-1β), Panel B is interleukin

6 (IL-6), Panel C is interleukin 10 (IL-10), Panel D is interleukin 17 (IL-17), Panel E is interferon gamma (INF-γ) and Panel F is tumor necrosis factor alpha (TNF-α). Wild type control mice were compared to the BTBR and VA group using a Student's t-test. The asterisk* denotes significance of p<.05.

Table 1. Statistical values for mouse models as compared to wild type control C57B/6J mice using serum taken from 21 day (P21) and 105 day old (P105) mice compared to the control group using an unpaired student t-test, significance is *p<.05.

Cytokine	BTBR P21	VA P21		BTBR P105	VA P105
Anti-inflammatory					
IL-10	.7880	.1883		.2729	.7177
Proinflammatory					
IL-1β	.0622	.1254		.0273	.4222
IL-6	.5943	.4103		.2205	.6079
IL-17	.7297	.2404		.4227	.3746
INFγ	.1410	.5208		.1925	.3966
TNF-α	.1362	.4330		.1896	.3143

Data trends found that all cytokines are increased in BTBR subjects compared to control in both P21 and P105 day mouse models, yet only IL-1β in P105 day mice was statistically significant (p=.02) when compared to control mice **(Figure 2, Panel A)**. In contrast, there was no significant increase or upward trend in any of the cytokines in the valproic acid subjects compared to the control in either age group.

Microbiome sequencing analyses

The CLC workbench software was used to performed alpha diversity, beta diversity, and phylogenetic diversity to assess variation within and between groups regarding species variation. Alpha diversity illustrates that species diversity or richness that is present within the samples. Observed operational taxonomic units (OTUs) was used to determine species diversity with the

groups **(Figure 3)**. A Kruskal Wallis test of nonparametric sampling was applied by the CLC workbench to determine if samples have the same distribution of species within the group. Analysis revealed that each group exhibited a unique species sampling based on OTU numbers. Both the BTBR (p=.002) and VA (p=.001) groups exhibited a significant different species sampling within the group.

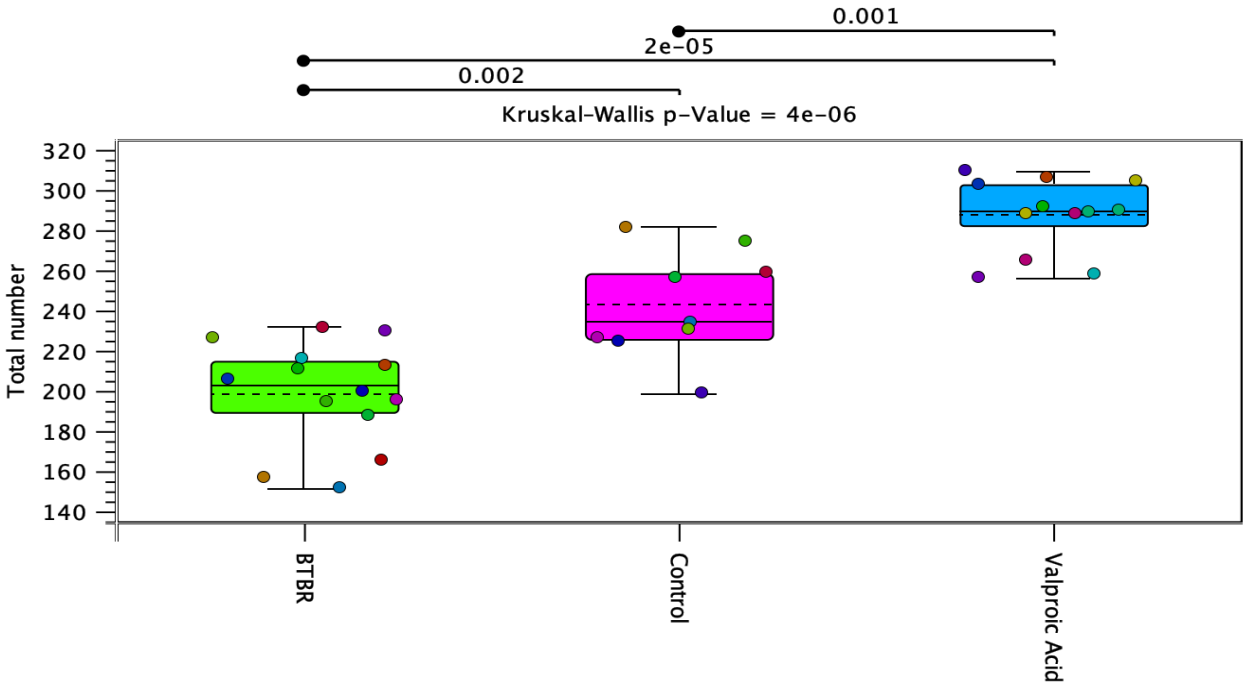

Figure 3. Alpha diversity across groups (Control N=6 green, BTBR N=8 pink, VA N=7 blue). Each individual sample that was analyzed is represented by a dot in its respective grouping. All p-values are less than 0.05, indicating that all groups are significantly different from the other, as shown by the p-value of <0.05. The BTBR group has the lowest richness, while valproic acid has the highest average richness across all groups and samples, as noted by placement of each group along the y-axis quantifying total number.

Phylogenetic diversity shows the measure of biodiversity per number of reads. For every sample present, phylogenetic diversity is above at least 2.4 and plateaus across number of reads. A phylogenetic diversity figure was also generated to assess species richness. As shown in **Figure 4**, the number of reads for all of the samples is the same, but the phylogenetic diversity is

different among all three test groups. BTBR are the lowest on the chart, indicating less phylogenetic diversity. Both control and valproic acid are higher than the BTBR group. The C57 control group and the VA group are shown around 4.2 instead of the 3.0 of BTBR. Samples rich in diversity will be closer to 5.

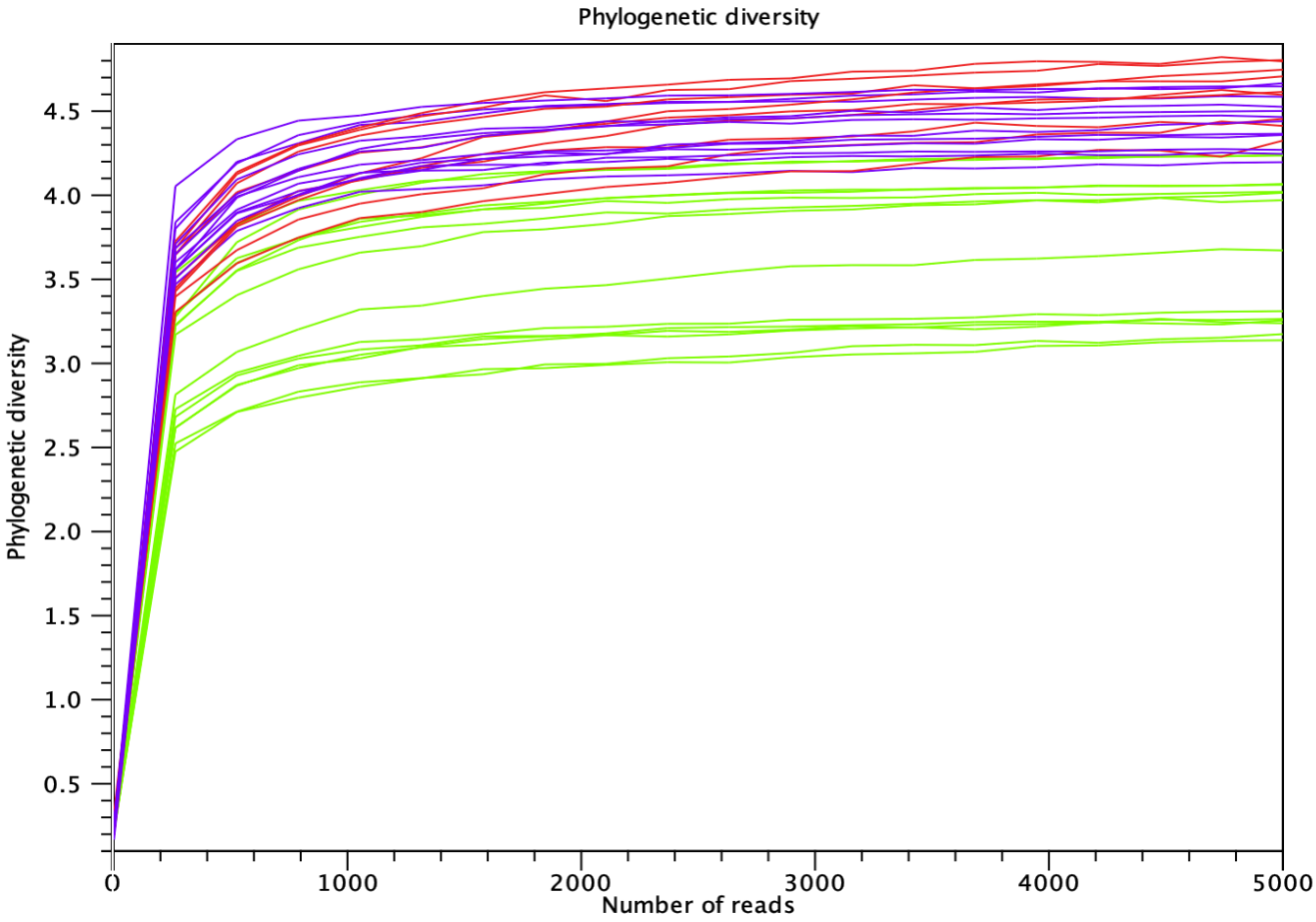

Figure 4. Phylogenetic Diversity between individual sample pairs where red lines are control, green lines indicate BTBR, and purple are valproic acid animals. The x-axis measures the number of reads for each sample. The y-axis measures phylogenetic diversity on a scale from 0-5. Data is displayed for control n=6, BTBR n=8, and VA n=7.

Beta diversity is a way to illustrate the difference in diversity across groups. A principal coordinate analysis is the actual measure of similarity between the groups. Each are a way to

determine the group differences with regard to OTUs. Both beta diversity and principal coordinate analysis were performed with visual representations displaying a clear separation that all samples in each group have compared to samples in other groups (**Figure 5A and 5B**). PCo1 and PCo2 show the largest percentages of variability (**Figure 5A**). If sample dots are close to each other on the scatterplot, this indicates that the composition in the samples is similar (Zhou et al., 2020). Panel B is the 3D version of beta diversity figure, and shows that same distinct separation between groups, but closeness in sample dots. Measures of beta diversity include a nonphylogenetic qualitative measure of dissimilarity between communities (**Appendix Table 2**, Jaccard p=<0.05) and measure of similarity between communities (**Appendix Table 1**, Bray-Curtis p=<0.05) that are both significantly different between the three communities corresponding to the three groups. The Unifrac, or quantitative distance between the communities using phylogenetic measures also indicated that the three groups were unique in species composition (**Appendix Table 3**, Unweighted Unifrac p=<0.05).

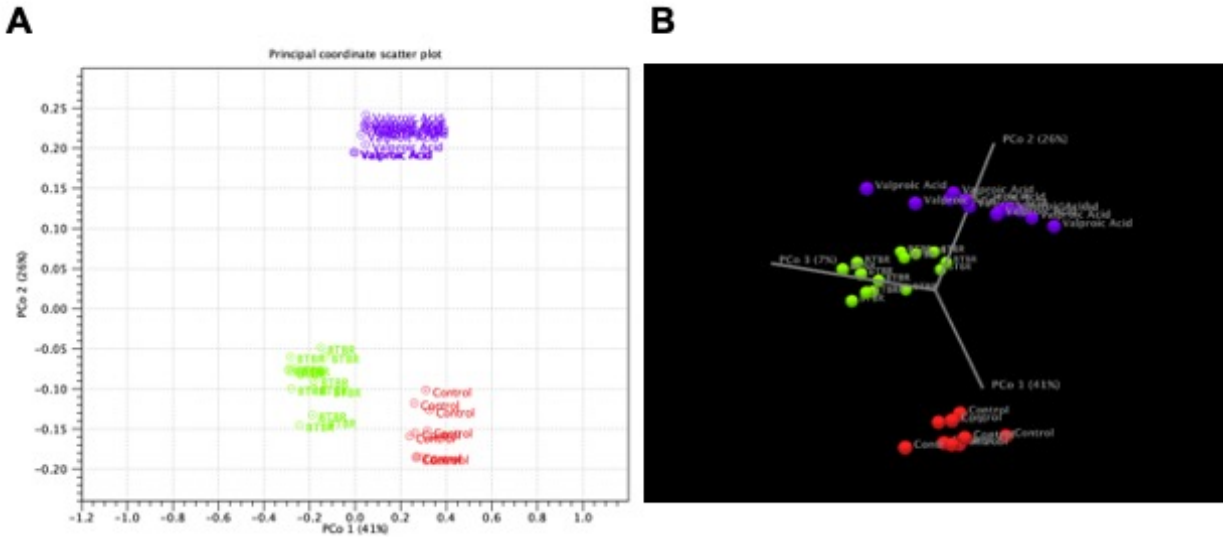

Figure 5. Panel A denotes the principal coordinate analysis (PCA) that is shown in in 2D form. Panel B is the 3D representation where red spheres represent the control group, green spheres indicate the BTBR group and purple are valproic acid animals.

A PERMANOVA, or permutational analysis of variance, is an analysis that compares all three groups side-by-side to determine significant differences in overall species expression between the groups. A PERMANOVA is similar to an ANOVA but has permutations to avoid any kind of skewing or bias in the sample set. Additionally, it is the most common type of distance-based method to test large groups with lots of OTUs, like microbial data (Tang et al., 2016). **Table 2** displays the PERMANOVA that was generated for this data set. All groups are compared to each other, and all of these comparisons yielded a p-value of <0.05, indicating that each group is significantly different from the other.

Table 2. PERMANOVA analysis comparing groups. A PERMANOVA was performed across all samples and groups, $p=<0.05$ for significance.

1 PERMANOVA analysis (D_0.5 UniFrac)

Variable	Groups	Pseudo-f statistic	p-value
Group	BTBR, Valproic Acid, Control	33.05970	0.00001

Group 1	Group 2	Pseudo-f statistic	p-value	p-value (Bonferroni)
BTBR	Valproic Acid	28.84250	0.00001	0.00003
BTBR	Control	38.97883	0.00001	0.00003
Valproic Acid	Control	31.84893	0.00001	0.00003

A heat map can be used to evaluate differences in distinct phyla groups by assigning color to expression patterns. This method of displaying data can be useful to simply glance at a set of data and report obvious qualitative phylogenetic expression differences shown between the groups. The heat map comparing the three groups of mice can be seen in **Figure 6** with observable qualitative differences present as indicated by the grouping of high and low expressing phyla.

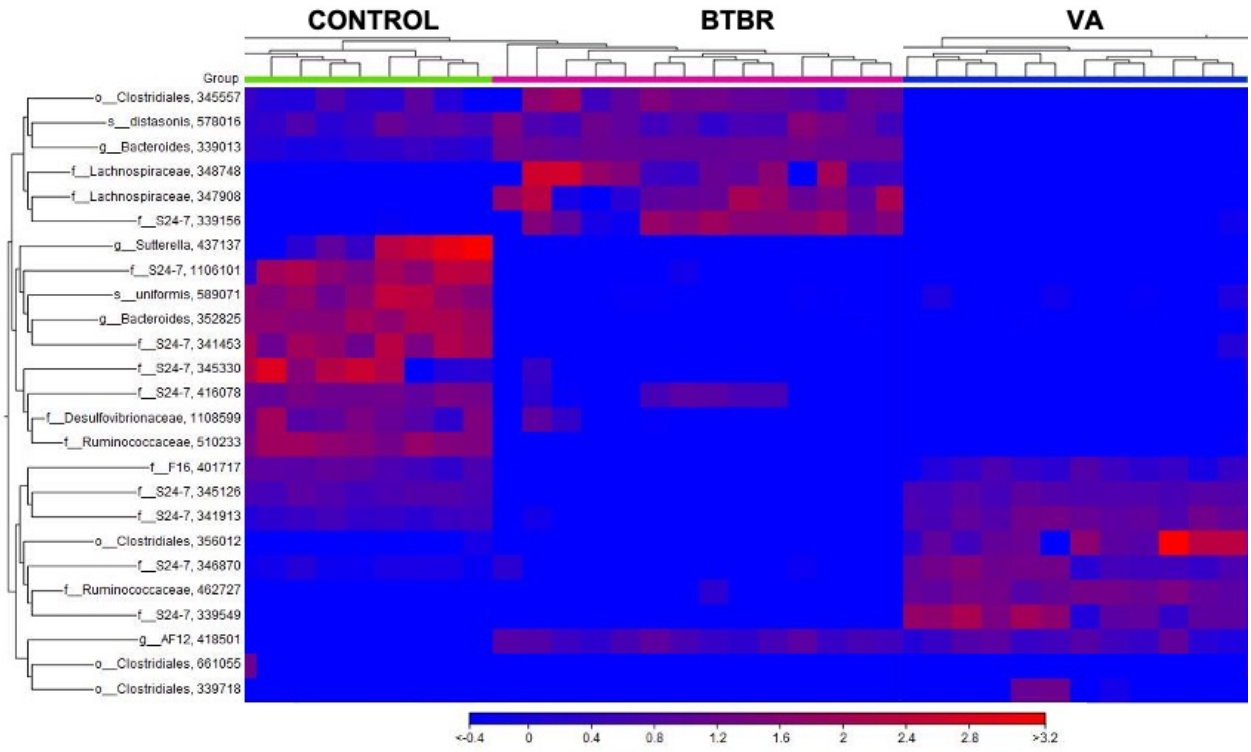

Figure 6. Heat map of bacterial species abundance across model groups. Species names are listed across the top of the X-axis and color coded with control (green bar), BTBR (pink bar) and VA (blue bar). Representative bacterial species names are listed down the side of the Y-axis. Red denotes a higher concentration of bacteria; blue denotes a lower concentration shown by the bar at the bottom of the image.

Bacterial phyla differences can also be visualized through specific taxonomic identification. Differences in order are shown between the mouse groups in **Figure 7**. Proteobacteria was reduced in both of the subject models compared to the wildtype control mice **(Figure 7)**. A Firmicutes to Bacteroides ratio was used to assess specific order differences among the groups that have been identified in human ASD. Ratios across model groups revealed a change in the amount of Bacteroides and Firmicutes in the BTBR model (**ratio =.25**) compared to the wildtype control **(ratio=.52)**, similar to the Firmicutes/Bacteroides ratio change noticed in

the Xie et al. study using Senescence-Accelerated Mouse Prone 8 (SAMP8) mouse models (Xie et al., 2020). However, the valproic acid ratio (**ratio=.66**) of Firmicutes/Bacteroides appeared to be more similar to the wild type control. Tenericutes are shown to be more prevalent in the BTBR group compared to valproic acid and the control based on the abundance levels from the graph.

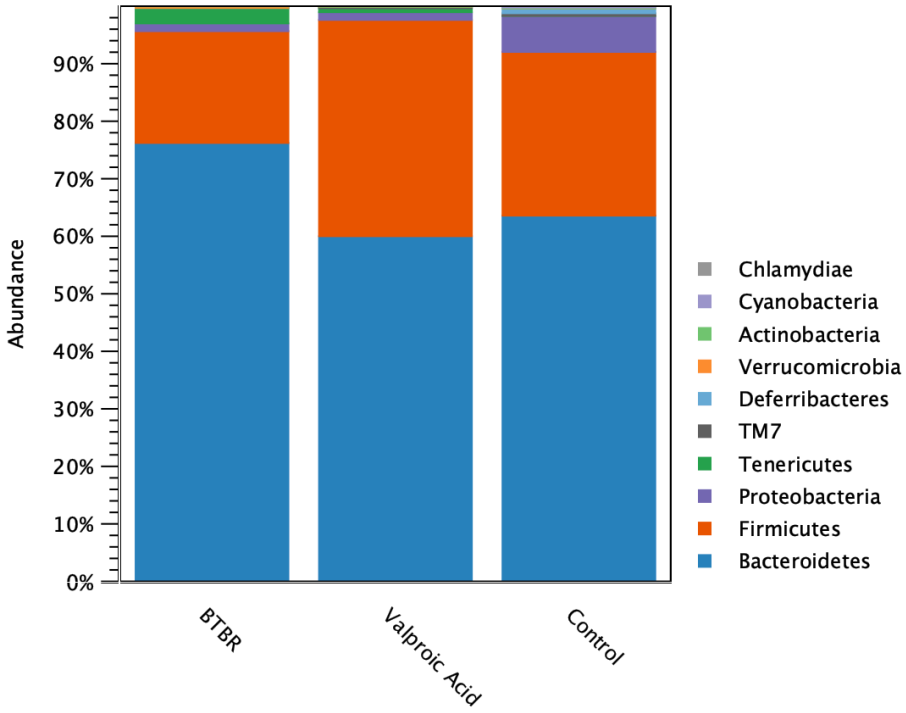

Figure 7. Differential abundance in bacterial species is shown for control C57/B6, BTBR and mice from valproic acid injected dams. In Figure 7, Bacteroides is denoted with a blue bar, firmicutes is denoted with an orange bar, and purple represents proteobacteria. Groups are denoted on the x-axis and relative abundance is shown along the y-axis. Firmicute-Bacteroides ratio was found to be 0.52 for control, 0.25 for BTBR, and 0.66 for valproic acid animals.

CHAPTER 4. DISCUSSION

Two proposed test mouse models were used in this study to determine if neuroinflammatory, peripheral cytokine, and gut microbiome data in humans could be recapitulated in these models. A genetic model, BTBR, and valproic acid-treated model were used and compared to the wildtype control mice. All of the data presented in this study are either novel to the literature or have been performed in only one other study for these models.

The hilum layer of the hippocampus and visual cortex areas were assessed in the BTBR and VA models as compared to control wild type mice for microglia activation. These areas have high axonal input from other brain regions and are in close proximately to the lateral ventricles. The ventricles have a rich blood supply and are found to contain areas of increased cell regeneration. Therefore, inflammatory molecules would likely cross in these regions to activate microglia and astrocytes. In the Matta et al 2019 study, increased microglial density was observed in the dentate gyrus of the Neuroligin-3 ($NL3^{R451C}$) along with increasing trends of microglial density in the hippocampus CA1 area (Matta et al., 2019). Interestingly, no significant differences in the microglia marker, IBA-1, were seen between the models in this research study. The study here did use the hilum of the hippocampus which is a projection area of pyramidal and mossy fiber neurons in the region. It is possible that microglia in this particular region are not activated in modifying dendritic elements of the large number of interneurons within this region. It might suggest that only microglia around the pyramidal cells are altered; therefore, the visual cortex immunohistochemistry was performed. Cortical region is composed of large numbers of pyramidal neurons when compared to the interneuron population in the same region. It was also found that the visual cortex did not demonstrate IBA-1 expression differences. These findings suggest that the activation found in the Neuroligin-3 ($NL3^{R451C}$) do

not extend to these brain regions or were marred by additional factors including microglia diversity. It is noted that IBA-1 is a global marker of inflammation and microglia have distinct phenotypes that respond to the different classes of cytokines, i.e. anti-inflammatory or proinflammatory.

Peripheral cytokines measured from serum are an available assessment of overall inflammation in a species. In ASD patients, it has been shown that cytokine expression is changed in some but not all patients. We postulated that cytokine levels in the animals would mimic some studies that have been shown in humans (Pettrelli et al., 2016). It was thought that if inflammation were present during gestation and continued into development, it could be identified through altered cytokine expression levels. Interestingly, only IL-1β in adult BTBR male group was significantly elevated. No differences were found in any of the young weanling groups for all measured inflammatory markers. Valproic acid trended towards increases in the adult P105 day males but did not make significance. Interestingly, IL-1β has an established relationship in ASD as shown through meta-analysis (Saghazadeh et al. 2019). It is extremely possible that IL-1β in ASD individuals could be interfering with overall brain development. IL-1β inflammation could be causing inadequate pruning in the brain, resulting in too many neurons and cells present. Inflammation would also result in stimulation of these neurons and cells, with no counteracting force present to halt this overabundance of stimulation and inflammation. IL-1β was recapitulated in the BTBR animal model, but only at the adult stage. If truly interfering with brain development, then it would have thought to be present in the weanling group that corresponds with children in human development.

Gut microbiota data indicated significant differences were present in bacterial composition of mouse models compared to a control model. Differential abundance shows a

significant decrease in proteobacteria in both BTBR and valproic acid mice compared to the C57 control group. Proteobacteria are primarily Gram-negative organisms that can potentially be pathogenic, however, can exist in certain amounts and contribute to a healthy gut (Moon et al., 2018). It is interesting that this phylum was decreased in both mouse models. It is possible that proteobacteria differences is the primary cause one reason of discomfort in the gastrointestinal tract. It is postulated that proteobacteria can consume oxygen, therefore eliciting an anaerobic environment where other members of normal gut flora can abound (Shin et al., 2015). It is reasonable to assume that overpopulation of proteobacteria would result in normal gut flora being depleted resulting in excessive oxygen content in the gastrointestinal tract directly resulting in bowel issues and discomfort in many ASD patients.

Firmicutes/Bacteroides ratio was also changed in the BTBR group. These phyla are the most abundant in both mice and humans and a ratio is a sufficient way to assess any imbalance in flora (Sauer et al., 2019). The valproic acid group and control group had similar ratios; these groups each had similar amounts of firmicutes and bacteroides, but BTBR exhibited a lack of firmicutes and increased amount of bacteroides. In the Rowin et al. study in 2017, the ratio of firmicutes and bacteroides in three of the five test subjects corresponded to intestinal inflammation and low microbe diversity (Rowin et al., 2017). Dissecting major sections of the relative abundance chart was not the focus of this study but is an important part future projects because each bacterial group plays an important role in normal gut flora. Any imbalance, like that mentioned above, can lead to inflammation and also the progression of neurological diseases and disorders like Alzheimer's and ASD (Rowin et al., 2017).

The C57 control group has a high concentration of Bacteroides and *Sutterella* spp. In contrast, the BTBR group had very low concentrations of the aforementioned species but showed

a higher concentration of *Clostridiales* group 345557 and *Lachnospiraceae* than both the C57 and the valproic acid groups. Lastly, valproic acid models were most highly concentrated in *Ruminococcaceae* and *Clostridiales* group 356012. All models within their respective groups have correlating bacterial species and concentrations, supporting the data exhibited in the beta diversity graph and described above—each of the three groups is very different from the other. Future studies using this data should determine if the specific taxonomic changes are correlated with ASD in human microbiome studies. Additionally, it will be interesting to determine what behavioral changes the taxa are associated within animals and humans.

All microbiota findings indicate that there are significant differences between the three groups for species richness and diversity. As displayed and mentioned above, each model group is notably different from one another. All individual animal samples that make up each group are similar in composition to one another i.e. all BTBR models have similar profiles. It would possibly be expected that BTBR would be dissimilar since it is a genetically inbred species. However, the valproic acid was unexpected due to the similarity between the two groups. The VA group are C57 mice with the only difference being a single injection of valproic acid during gestation of the VA offspring. The IBA-1 and cytokine data did not indicate any differences between the C57 control group or the VA group, but the microbiome data was vastly different. This would indicate that quite possibly the differences that are found in the literature for the VA group when compared to the C57 control group are working through a unique mechanism that was not identified through the current markers.

Limitations

Limitations in these studies could be found. For example, this was a pilot evaluation to determine if these models are useful in future studies on treatments for social behavior, anxiety or sensory deficits so a small study number of animals was utilized for much of the experimentation. More samples are needed to determine if trends are accurate for the findings. Another limitation exists in the BTBR as it is a genetically inbred model group. While related to the C57 strain of mice, there is not a validated, accepted control mouse for this group and it is debated whether the C57 is an appropriate control. However, it is used throughout the literature as a control mouse for the BTBR inbred strain. Furthermore, this preliminary study only focused on male mouse data. No female studies were included in this study. Though ASD is typically more prevalent in males, it would be interesting to have determined if findings extend to females. Also, immunohistochemical findings only utilized one global marker for microglia activation. Additionally, astrocytes can also influence cytokine production in the brain and were not examined in these studies.

Conclusion

Taken together, these studies revealed some interesting findings. Gut microbiome data yielded vastly different profiles between each of the three groups. Overall, only one cytokine was significantly changed, and there were no significant changes in IBA-1 immunohistochemical staining data. It is tempting to speculate that the BTBR and valproic acid models used here are not ideal mouse models in studying ASD-like pathology. However, it is possible that the gut microbiome may be signaling through another mechanism to induce behavior deficits that have

been recorded in the literature. Further research is needed to determine suitable animal models for ASD research, specifically for peripheral and neuroinflammation.

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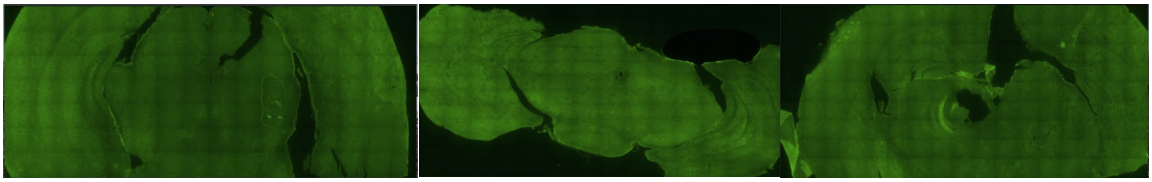
APPENDIX: Supplementary Materials

Appendix Figure 1. GFP images of IBA-1 staining representative of a batch. Staining and imaging was performed in batches.

C2183 B5013 V446



Appendix Table 1. Bray-Curtis PERMANOVA Analysis of mouse model groups; $p<0.05$ indicates significance.

1. PERMANOVA analysis (Bray-Curtis)

Variable	Groups	Pseudo-f statistic	p-value
Group	BTBR, Control, Poly I:C, Valproic Acid	23.36279	0.00001

Group 1	Group 2	Pseudo-f statistic	p-value	p-value (Bonferroni)
BTBR	Control	42.70179	0.00001	0.00006
BTBR	Poly I:C	22.15391	0.00033	0.00196
Control	Poly I:C	15.12462	0.00140	0.00839
BTBR	Valproic Acid	25.10087	0.00001	0.00006
Control	Valproic Acid	25.88829	0.00001	0.00006
Poly I:C	Valproic Acid	6.27550	0.00055	0.00330

Appendix Table 2. Jaccard PERMANOVA analysis of mouse model groups; $p<0.05$ indicates significance.

2. PERMANOVA analysis (Jaccard)

Variable	Groups	Pseudo-f statistic	p-value
Group	BTBR, Control, Poly I:C, Valproic Acid	13.09531	0.00001

Group 1	Group 2	Pseudo-f statistic	p-value	p-value (Bonferroni)
BTBR	Control	21.22969	0.00001	0.00006
BTBR	Poly I:C	11.85899	0.00033	0.00196
Control	Poly I:C	8.47335	0.00140	0.00839
BTBR	Valproic Acid	15.14397	0.00001	0.00006
Control	Valproic Acid	13.99128	0.00001	0.00006
Poly I:C	Valproic Acid	4.55169	0.00055	0.00330

Appendix Table 3. Unweighted UniFrac PERMANOVA analysis of mouse model groups; $p<0.05$ indicates significance.

4. PERMANOVA analysis (Unweighted UniFrac)

Variable	Groups	Pseudo-f statistic	p-value
Group	BTBR, Control, Poly I:C, Valproic Acid	47.05406	0.00001

Group 1	Group 2	Pseudo-f statistic	p-value	p-value (Bonferroni)
BTBR	Control	55.07413	0.00001	0.00006
BTBR	Poly I:C	31.46685	0.00033	0.00196
Control	Poly I:C	20.06766	0.00140	0.00839
BTBR	Valproic Acid	53.61046	0.00001	0.00006
Control	Valproic Acid	76.56428	0.00001	0.00006
Poly I:C	Valproic Acid	22.17955	0.00055	0.00330

Appendix Table 4. OTU differential abundance analysis, C57 wildtype.

OTU (Table) (differential abundance analysis)-1

Name	Max group mean
o__Clostridiales, 349874	46.71
o__Clostridiales, 173739	12.36
g__Oscillospira, 307608	3.33
o__Clostridiales, 262625	1.33
f__Ruminococcaceae, 266720	7.86
g__Clostridium, 234912	0.14
f__S24-7, 177512	0.44
g__Coprococcus, 339791	0.21
g__Bacteroides, 2198356	0.36
o__Clostridiales, 319219	0.14
f__Lachnospiraceae, 196533	0.11
g__Oscillospira, 328905	0.11
g__Clostridium, 1105343	0.11
o__Clostridiales, 192475	10.43
o__Clostridiales, 129394	19.14
f__Ruminococcaceae, 1105016	2.71
f__Ruminococcaceae, 269156	4.71
o__Clostridiales, 174358	15.86
g__Ruminococcus, 3016478	3.36
g__Oscillospira, 263337	2.56
o__Clostridiales, 290338	1.14
f__Lachnospiraceae, 348748	12.57
o__Clostridiales, 157154	1.00
o__Clostridiales, 356590	0.57
o__Clostridiales, 827195	1.71
f__Ruminococcaceae, 1110253	3.79
f__Lachnospiraceae, 259580	2.14
f__S24-7, 258530	15.33
f__Lachnospiraceae, 305177	33.57
f__Ruminococcaceae, 265793	3.21
o__Clostridiales, 190652	0.00
o__Clostridiales, 324522	2.79
f__Lachnospiraceae, 347908	7.14

Appendix Table 5. OTU differential abundance analysis, BTBR.

OTU (Table) (differential abundance analysis)-1

Name	Max group mean
o__Clostridiales, 349874	46.71
o__Clostridiales, 173739	12.36
g__Oscillospira, 307608	3.33
o__Clostridiales, 262625	1.33
f__Ruminococcaceae, 266720	7.86
g__Clostridium, 234912	0.14
f__S24-7, 177512	0.44
g__Coprococcus, 339791	0.21
g__Bacteroides, 2198356	0.36
o__Clostridiales, 319219	0.14
f__Lachnospiraceae, 196533	0.11
g__Oscillospira, 328905	0.11
g__Clostridium, 1105343	0.11
o__Clostridiales, 192475	10.43
o__Clostridiales, 129394	19.14
f__Ruminococcaceae, 1105016	2.71
f__Ruminococcaceae, 269156	4.71
o__Clostridiales, 174358	15.86
g__Ruminococcus, 3016478	3.36
g__Oscillospira, 263337	2.56
o__Clostridiales, 290338	1.14
f__Lachnospiraceae, 348748	12.57
o__Clostridiales, 157154	1.00
o__Clostridiales, 356590	0.57
o__Clostridiales, 827195	1.71
f__Ruminococcaceae, 1110253	3.79
f__Lachnospiraceae, 259580	2.14
f__S24-7, 258530	15.33
f__Lachnospiraceae, 305177	33.57
f__Ruminococcaceae, 265793	3.21
o__Clostridiales, 190652	0.00
o__Clostridiales, 324522	2.79
f__Lachnospiraceae, 347908	7.14

Appendix Table 6. OTU differential abundance analysis, valproic acid.

OTU (Table) (differential abundance analysis)-1

Name	Max group mean
o__Clostridiales, 349874	11.00
o__Clostridiales, 173739	3.08
g__Oscillospira, 307608	6.42
o__Clostridiales, 262625	2.17
f__Ruminococcaceae, 266720	4.92
g__Clostridium, 234912	0.25
f__S24-7, 177512	0.83
g__Coprococcus, 339791	0.25
g__Bacteroides, 2198356	0.25
o__Clostridiales, 319219	0.25
f__Lachnospiraceae, 196533	0.25
g__Oscillospira, 328905	0.25
g__Clostridium, 1105343	0.25
o__Clostridiales, 192475	7.58
o__Clostridiales, 129394	4.00
f__Ruminococcaceae, 1105016	0.00
f__Ruminococcaceae, 269156	0.00
o__Clostridiales, 174358	0.00
g__Ruminococcus, 3016478	0.00
g__Oscillospira, 263337	5.08
o__Clostridiales, 290338	0.00
f__Lachnospiraceae, 348748	0.00
o__Clostridiales, 157154	0.00
o__Clostridiales, 356590	0.00
o__Clostridiales, 827195	0.00
f__Ruminococcaceae, 1110253	0.00
f__Lachnospiraceae, 259580	0.00
f__S24-7, 258530	27.67
f__Lachnospiraceae, 305177	0.00
f__Ruminococcaceae, 265793	0.00
o__Clostridiales, 190652	0.00
o__Clostridiales, 324522	0.00
f__Lachnospiraceae, 347908	0.00

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