12-2019

Cannabidiol, behavior, and immune biomarkers in idiopathic autism spectrum disorder.

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CANNABIDIOL, BEHAVIOR, AND IMMUNE BIOMARKERS

IN IDIOPATHIC AUTISM SPECTRUM DISORDER

By

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B.A. Comparative Literature, Brown University, 2015
M.S. Pharmacology and Toxicology, University of Louisville, 2019

A Dissertation
Submitted to the Faculty of the
School of Medicine of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy
in Pharmacology and Toxicology

Department of Pharmacology and Toxicology
University of Louisville
Louisville, Kentucky

December 2023

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A Dissertation Approved on

November 21, 2023

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DEDICATION

This thesis is dedicated to my grandparents

Edward C. Shrader and Gloria Riebel Shrader

&

K.C. Huang and Shou Shan Huang

who instilled in me the importance of life-long education and provided

the moral, intellectual, and financial means to pursue my passion for science.

Their sacrifices and love have been instrumental in forming

the woman and scientist I am today.

ACKNOWLEDGEMENTS

I would like to thank my mentor Dr. Zhao-Hui Song, who has believed in me since day one, stood by me through the many failures, and encouraged all of my scientific pursuits. I would also like to thank my co-mentor Dr. Gregory Barnes, who has helped to foster my interest in autism and to pursue a career dedicated to neuroscience research. Many thanks to my parents Dr. Robb Shrader and Dr. Anna Huang Shrader for the many forms of support they have lovingly shown throughout my time as a graduate student. Lastly, I want to extend my thanks to my friends Anna Zeidman, Ada Cahill, Doug Saforo, William Gibson & Müge Sak for providing me the emotional strength to persevere through it all.

ABSTRACT

CANNABIDIOL, BEHAVIOR, AND THE IMMUNE SYSTEM

IN IDIOPATHIC AUTISM SPECTRUM DISORDER

Sarah Huang Shrader

November 21, 2023

Autism Spectrum Disorder (ASD) is a highly heterogenous neurodevelopmental disorder currently estimated by the CDC to affect 1 in 36 children in the U.S.. This uniquely human condition is characterized by a spectrum of symptoms that feature social communication deficits and repetitive behaviors with restricted interest. Autistic individuals commonly exhibit multiple comorbidities, including attention deficit hyperactivity disorder, anxiety, and seizures, which can complicate an already multi-faceted presentation.

The majority of ASD cases are idiopathic in nature. Developing a well-validated animal model of ASD for translational research comes with many challenges, but is crucial for advancing our understanding of autism and developing therapeutic interventions. In this dissertation, the BTBR mouse model of idiopathic autism was chosen, due to its strong behavioral and immunological face validity.

A pharmacological intervention for the treatment of core autistic symptoms has yet to be identified. While cannabidiol (CBD), the major nonpsychoactive constituent of *Cannabis sativa*, is suggested to have multiple therapeutic applications, its effect(s) on

idiopathic autism remains unclear. We hypothesized that chronic CBD treatment would effectively attenuate the autism-like behaviors observed in BTBR mice. Weanlings were injected daily with either vehicle, 20 mg/kg CBD or 50 mg/kg CBD for two weeks, and subsequently assessed with a battery of behavioral assays. Our data indicate that the therapeutic effects of CBD on specific behaviors of BTBR mice are dose-dependent, with high dose CBD treatment attenuating repetitive self-grooming behavior and hyperlocomotion, and low dose CBD rescuing sociability deficits.

Gaining a deeper understanding of the neurobiological and immunological underpinnings of idiopathic ASD is essential to the identification of new diagnostic and therapeutic approaches to autism. We sought to characterize the BTBR immune profile using flow cytometric analysis of blood, peripheral lymphoid tissues, and whole brain samples. Our data demonstrate, for the first time, alterations in the peripheral γδ T cell profile and microglial expression of TREM2, both of which have been implicated in clinical ASD.

Collectively, this dissertation highlights CBD's efficacy as a pharmacological intervention for the treatment of core and co-morbid ASD symptoms and identifies γδ T cells and TREM2 expression as potential immunological biomarkers for ASD diagnosis.

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INTRODUCTION

I. Autism Spectrum Disorder

Autism Spectrum Disorder (ASD) is a complex and heterogeneous neurodevelopmental condition that affects social communication, repetitive behavior, and sensory processing [1]. The term "spectrum" reflects the wide variability in symptoms and severity, making each individual with ASD unique in their presentation. Over the past two decades, the prevalence of ASD has seen a significant increase, now affecting an estimated 1 in 36 children [2]. With ASD gradually becoming one of the most common neurodevelopmental disorders, the rise in cases has significant implications for healthcare, education, and society as a whole. This increasing prevalence may be partially attributed to several factors, including increased awareness, improved diagnostic criteria, and a broader understanding of the disorder [3, 4]. However, some researchers argue that these elements alone cannot fully explain the growing frequency of individuals with autism [5, 6]. Ongoing research is crucial to better understand the complex interplay of genetic and environmental factors in ASD and to develop more effective interventions and support for individuals and families affected by the disorder.

1.1. Etiology

Over the years, our understanding of ASD has evolved, with preclinical, clinical and epidemiological research shedding light on its complex etiology involving diverse genetic and environmental risk factors [7-10].

Genetic Factors: Genetic studies have identified a strong hereditary component in ASD. Identical twins are more likely to both have autism compared to non-identical twins, and the risk of autism is higher in families with a history of the disorder [11, 12]. Genome-wide association studies have identified gene clusters with common themes converging on signaling pathways involved in immune system function, synaptic dysfunction and brain development [13]. Although the majority of cases are idiopathic or polygenic, about ~10-25% can be attributed to rare genetic variants including *de novo* mutations and monogenic disorders, such as Fragile X Syndrome, Tuberous Sclerosis Complex, and Rett Syndrome [11, 12]. Studies into syndromic autism have aided in the identification of specific gene mutations and variations that may prove to be useful in developing therapeutic targets for pharmacological intervention [11, 12].

Environmental Factors: Environmental factors are able to induce epigenetic alterations that can influence gene expression, which may have a large impact on development and the pathogenesis of ASD. Research have identified a number of prenatal risk factors for ASD, including parental age, birth order, maternal physical and mental health, maternal prenatal medication use and familial socioeconomic status [14]. Natal and postnatal risk factors such as abnormal gestational age, hypoxia, early infant infection and air pollution exposure may also increase a child's susceptibility to autism [14]. During each stage of development pre- and post-birth, environmental factors, when combined with genetic alterations, are thus thought to play significant roles in the increased risk of developing idiopathic ASD.

Figure 1 illustrates how the interactions between these genetic and environmental risk factors are thought to contribute to the underlying mechanisms leading to ASD, and how they may shape our approach to identifying potential diagnostic biomarkers.

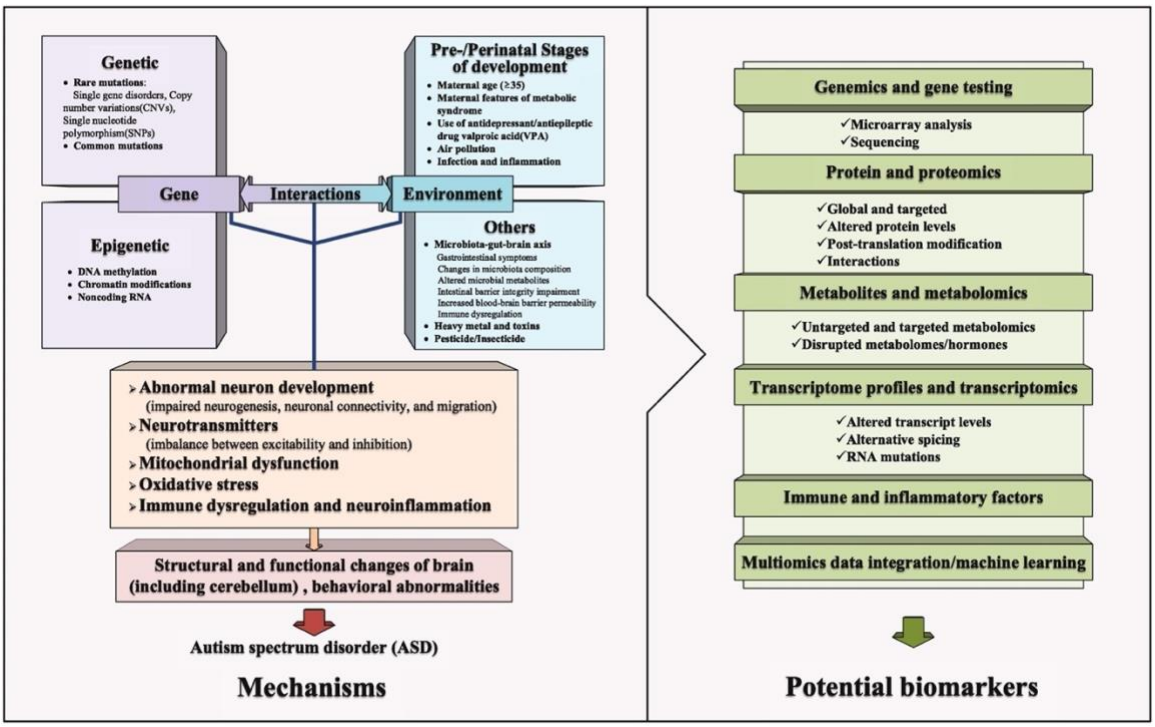

Figure 1. Proposed mechanisms associated with ASD and potential diagnostic biomarkers

ASD is thought to be a multifactorial disorder rooted in complex interactions between genetic and environmental risk factors. The genetic aspect encompasses both traditional genetic and epigenetic factors, such as single gene mutations, copy number variants (CNVs), DNA methylation and chromatin modifications. The environmental aspect involves factors related to pre- and post-natal developmental stages, the microbiota-gut-brain axis, and exposure to toxicants. These genetic and environmental factors can collectively contribute to physiological alterations, including abnormal neuron development, alterations in neurotransmitters, mitochondrial dysfunction, oxidative stress, immune dysregulation, and neuroinflammation. Together, these changes can impact the structure and function of the brain, ultimately leading to the development of

ASD. With these underlying mechanisms and physiological alterations in mind, researchers are investigating potential diagnostic biomarkers using various genetic, proteomic, metabolomic, transcriptomic, and immunological methods. This figure is adapted from Shen et al. (2020) [15].

1.2. Clinical Presentation

ASD is characterized by a triad of core symptoms, which include deficits in social communication, repetitive behaviors, and restricted interests [16, 17]. Autism typically manifests in early childhood, and most core symptoms become evident between the ages of 12 to 24 months [18]. Clinical features vary widely and may include language delays, sensory sensitivities, and challenges in understanding and expressing emotions. The three core impairments often co-occur with a variety of comorbid conditions that can further impact the lives of individuals with ASD.

Core Symptoms of ASD:

Impaired Social Interaction: One of the hallmark symptoms of ASD is a difficulty in understanding and engaging in social interactions. Individuals with ASD may struggle with making eye contact, reading facial expressions, and understanding social cues. They often find it challenging to form and maintain relationships.

Communication Difficulties: Impaired communication is another core symptom of ASD. Some individuals may have delayed speech development, while others may never develop functional speech. Many individuals with ASD rely on alternative communication methods, such as sign language or communication devices. Additionally, individuals with ASD may struggle with understanding and using language pragmatics, making it challenging for them to engage in meaningful conversations.

Repetitive Behaviors and Restricted Interests: Autistic individuals often engage in repetitive behaviors, such as hand-flapping, rocking, or lining up objects. Some tend to have highly focused and intense interests in specific topics, often to the exclusion of other

activities. These behaviors and interests can provide comfort and predictability but may interfere with daily functioning.

Co-occurring Conditions and Comorbidities:

Intellectual and Developmental Disabilities: Many individuals with ASD have comorbid intellectual and developmental disabilities. This means they may have below-average intellectual functioning and difficulties in adaptive behaviors, such as daily living skills.

Attention-Deficit/Hyperactivity Disorder (ADHD): ADHD is commonly comorbid with ASD. Individuals with ADHD may have difficulty with attention, impulse control, and hyperactivity. The presence of both conditions can make it even more challenging to focus and complete tasks.

Anxiety and Mood Disorders: Anxiety disorders, including generalized anxiety, social anxiety, and obsessive-compulsive disorder, are frequently seen in individuals with ASD. Mood disorders like depression may also occur, especially in those who face difficulties in social interactions and communication.

Epilepsy: Epilepsy is a neurological disorder characterized by recurrent seizures. It is more prevalent in individuals with ASD than in the general population, and the presence of epilepsy can complicate the management of ASD symptoms.

Sensory Processing Difficulties: Many individuals with ASD have sensory processing difficulties, which can lead to hypersensitivity or hyposensitivity to sensory stimuli like light, sound, touch, and taste. These sensory challenges can cause discomfort and contribute to behaviors like avoidance or self-stimulation.

Gastrointestinal Issues: Some individuals with ASD experience gastrointestinal problems, including abdominal pain, constipation, and diarrhea. The relationship between gastrointestinal issues and ASD is still a subject of research and debate.

1.3. Diagnosis

As there are no genetic, neuroimaging, or electrophysiological tests for the definitive diagnosis of ASD, physicians have relied on comprehensive clinical assessments that involve observing and characterizing behavioral, social, and cognitive patterns [19, 20]. Prior to 2013, patients were diagnosed with one of five disorders that fell under the umbrella grouping of Pervasive Developmental Disorders: Autistic Disorder, Asperger's Disorder, Rett's Disorder, Pervasive Developmental Disorder–Not Otherwise Specified (PDD-NOS), and Childhood Disintegrative Disorder (CDD) [16, 21]. The revisions implemented in the most recent edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) removed these subcategories, focusing instead on two core domains: difficulties in social communication and interaction, and restricted and repetitive behaviors or interests [16]. In doing so, this broadened the definition to encompass the wide variation of symptoms experienced by patients and enabled the identification of individuals with ASD, who previously went unsupported and untreated [16, 19, 20].

The diagnosis of Autism Spectrum Disorder (ASD) is typically made by healthcare professionals with expertise in developmental disorders, such as pediatricians, child psychiatrists, or clinical psychologists. Given the early onset of the condition, healthcare professionals and researchers stress the critical importance of early diagnosis

for long-term patient outcome. The diagnostic process usually involves a comprehensive assessment that includes several components [16, 19, 20]:

1. *Clinical Evaluation:* The first step is often a clinical evaluation, during which the healthcare professional interviews the individual and their parents or caregivers. They will ask questions about the individual's developmental history, behavior, and any concerns related to social communication, repetitive behaviors, and sensory sensitivities.
2. *Observation and Interaction:* The healthcare professional will observe and interact with the individual to assess their social communication skills, behavior, and overall development. They may use structured play or interaction tasks to evaluate the individual's ability to engage with others, understand social cues, and communicate.
3. *Developmental and Behavioral Assessment:* Standardized assessment tools and questionnaires, such as the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview - Revised (ADI-R), are often used to gather additional information. These tools help to quantify and standardize observations and responses. Advances in early detection tools, such as the Modified Checklist for Autism in Toddlers (M-CHAT) and the use of developmental screening in pediatric care, have improved the recognition of ASD in young children.
4. *Medical Assessment:* A medical assessment is important to rule out any medical conditions or genetic syndromes that may mimic autism or co-occur with it. This may include physical and neurological examinations, genetic testing, and metabolic screenings.

5. *Parent and Caregiver Input:* Parents and caregivers play a crucial role in the diagnostic process by providing information about the individual's behavior, development, and any concerns they may have.
6. *Educational Assessment:* For children in school, an educational assessment may also be conducted to evaluate their educational needs and to help plan appropriate interventions.
7. *Multidisciplinary Team:* In some cases, a multidisciplinary team of specialists, including speech therapists, occupational therapists, and developmental pediatricians, may be involved in the evaluation and diagnosis.

Furthermore, diagnosing ASD is not without its challenges due to the presence of comorbidities, such as ADHD and anxiety disorders [6, 22]. These co-occurring conditions introduce complexity both in the diagnostic process and in determining the most suitable treatment strategies. Managing these comorbidities alongside ASD is an essential consideration in providing comprehensive care for individuals with these conditions. It highlights the need for a holistic and individualized approach to diagnosis and treatment, recognizing the unique needs and challenges faced by each patient.

1.4. Treatment and Intervention

A growing body of evidence supports the idea that early intervention can have a substantial positive impact on the long-term outcomes of individuals with ASD [18, 23-25]. Detecting and addressing the condition at an early stage can lead to more effective interventions and improved developmental trajectories. Treatment plans are often

developed in collaboration with healthcare professionals, educators, and therapists to provide a holistic and comprehensive approach to care that is individualized to meet the specific needs and strengths of each person with autism.

Behavioral and Educational Therapies: Early intervention programs, such as Applied Behavior Analysis (ABA) and speech therapy, can significantly improve the social and communication skills of children with ASD [26]. Individualized education plans (IEPs) in school settings are also important [26].

Medications: Currently, no pharmacological therapy for the treatment of core ASD symptoms has been developed. However, medications may be prescribed to manage specific symptoms associated with ASD [6]. These may include antipsychotics like risperidone or aripiprazole for aggression, selective serotonin reuptake inhibitors (SSRIs) like fluoxetine or sertraline for anxiety and depression, and stimulants like methylphenidate or atomoxetine for hyperactivity [6].

Complementary and Alternative Interventions: Some families explore complementary and alternative treatments, such as dietary interventions, sensory integration therapy, and social skills groups [17]. The effectiveness of these approaches varies, and caution is advised.

Ongoing research: At present, therapeutic intervention for ASD focus on alleviating symptoms, rather than targeting the underlying etiology. ongoing research is vital to better understand the most effective interventions and to continually improve outcomes for individuals with ASD. While the genetic heterogeneity presents clear challenges in the isolating specific therapeutic targets, researchers have suggested

diagnostic and treatment biomarkers relating to immunological, metabolic, and neurophysiological processes [27-30].

1.5. Male Preponderance

One of the most notable and consistent findings within the field of ASD is the higher prevalence of autism in males compared to females, with an average sex ratio of 4.2 to 1 [2]. However, the cause(s) for this male preponderance is not well understood. As such, the pronounced sex differences in regards to neurobiology, diagnosis, and behavioral presentation have been a topic of much discussion among clinicians and researchers [31-34].

Some genetic studies have identified sex-specific genetic variants associated with autism [31-34]. It is possible that these genetic factors interact with sex hormones to influence the development of the condition. For example, high levels of testosterone in the womb have been linked to an increased risk of autism in males. Researchers have also proposed that females may possess genetic or biological protective factors that make them less susceptible to developing autism or that they require a greater genetic burden to manifest the condition.

The diagnostic criteria for autism have traditionally been based on male-centered symptomatology [31-34]. Some research suggests that females with autism may present with somewhat different symptom profiles compared to males. For instance, they may exhibit better social communication skills and fewer stereotyped or repetitive behaviors, which could make it more challenging to recognize autism in females. This has led to concerns about diagnostic biases and resulting underdiagnosis in females. Growing

awareness of the possibility of autism in females and efforts to improve the diagnostic criteria for ASD may help reduce the gender disparity in autism diagnosis in the future.

II. Rodent Models of Autism Spectrum Disorder

Understanding the underlying mechanisms and potential treatments for ASD has been a significant challenge for researchers due to its heterogeneous nature. Mouse models have emerged as valuable tools in the study of ASD, providing insights into the genetic, environmental, neurobiological, and behavioral aspects of the disorder [35-37]. Figure 2 illustrates the role of these animal model in translational cycle of ASD research.

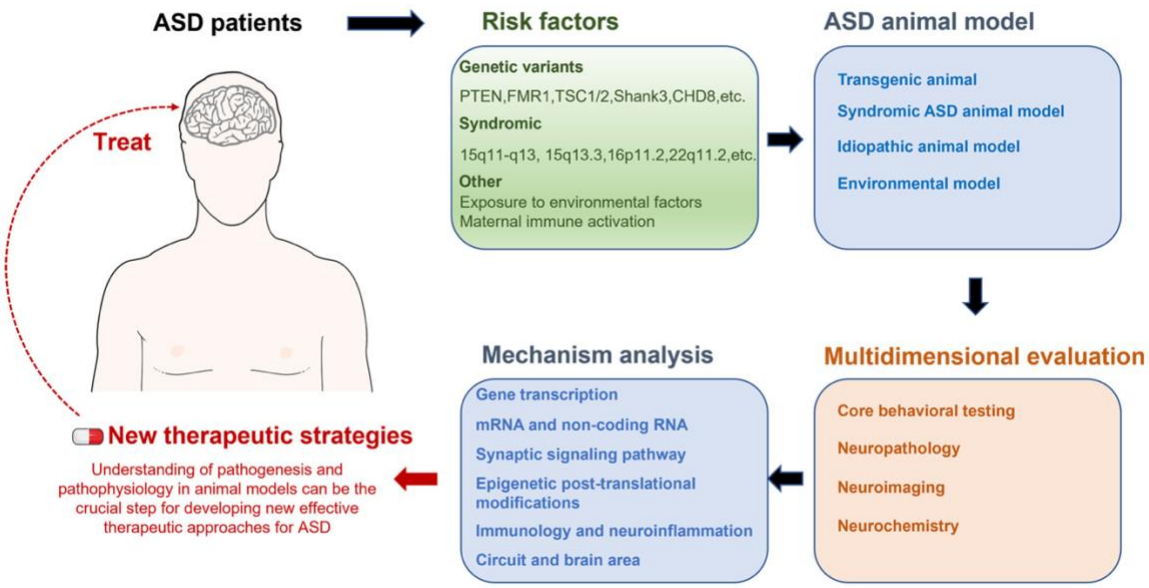

Figure 2. The role of animal models in translational ASD research

Epidemiological, GWAS and familial studies of autistic patients have led to the identification of various genetic and environmental risk factors implicated in clinical ASD. Based on these findings, different animal models have been developed, including monogenic, idiopathic and environmental models. Multidimensional evaluation of these animal models can lead to a deeper understanding of the mechanisms underlying ASD pathogenesis. Together, a comprehensive analysis of findings in both animal models and human patients may hopefully open avenues for identifying novel therapeutic strategies and pharmacological interventions that are clinically effective. This figure is adapted from Wang et al. (2023) [38].

2.1. Genetic Mouse Models of ASD

Genetic mouse models of autism have revolutionized our understanding of the genetic underpinnings of ASD. These models mimic various genetic mutations associated with the disorder, enabling researchers to explore the molecular and neural mechanisms that lead to ASD-like behaviors.

2.1.1 Fragile X Syndrome Mouse Model

Fragile X Syndrome (FXS) is the leading cause of monogenetic autism and is characterized by intellectual disabilities, social and behavioral challenges, and a range of physical and cognitive symptoms [39, 40]. Approximately 30% of patients with FXS are diagnosed with ASD [41]. FXS is caused by a mutation in the Fragile X Mental Retardation 1 (FMR1) gene, which leads to a deficiency in the Fragile X Mental Retardation Protein (FMRP) [39, 40]. In FXS, the absence of FMRP disrupts normal neuronal development and functioning, leading to an imbalance between excitatory and inhibitory synaptic transmission [39]. There is an overabundance of excitatory signals, which can contribute to hypersensitivity and excessive neural activity, along with a deficiency in inhibitory signals, which can lead to impaired learning and cognitive deficits [39]. FMRP normally plays a role in synaptic plasticity, the ability of synapses to strengthen or weaken in response to learning and experience [39]. Without FMRP, the brain's ability to adapt and reorganize itself in response to learning and environmental stimuli is compromised [39]. The molecular disruptions in FXS have a direct impact on the cognitive and behavioral symptoms of the syndrome, including intellectual disabilities, social and communication difficulties, and repetitive behaviors [39].

The *Fmr1* knockout (*Fmr1* KO) mouse is one of the most well-studied genetic mouse models of ASD, as it exhibits several characteristics and behaviors that resemble the features of human FXS [37, 42]. These include cognitive deficits, behavioral abnormalities, social deficits, and altered sensory processing [37]. The behavioral phenotypes of the *Fmr1* KO mouse model are often used to study the impact of FMRP deficiency on the nervous system and behavior, as well as on brain development and synaptic plasticity, shedding light on the synaptic dysfunction seen in ASD [37, 42]. Similar to in humans, in *Fmr1* KO mice, there is an imbalance between excitatory and inhibitory neurotransmission in the brain, which can lead to the hyperexcitability of neural circuits and impairments in learning and memory [37, 42].

2.1.2 Shank3 Mutation Mouse Model

SHANK3 is a synaptic scaffold protein associated with Phelan-McDermid syndrome, which can cause severe expressive speech and language delay, global developmental delay, epilepsy and ASD [43, 44]. The Shank3 gene, also known as SH3 and multiple ankyrin repeat domains 3, is located in the 22q13.3 region of the human genome, and plays an important role in synaptic transmission and plasticity [43].

Researchers have developed mouse models with mutations in the Shank3 gene to mimic the genetic disruptions seen in humans [45]. These models typically involve the deletion, mutation, or knockout of the Shank3 gene in mice, leading to the loss or dysfunction of the Shank3 protein in the mouse brain [45]. Mouse models with Shank3 mutations display ASD-like behaviors, including impaired social interaction and communication [45, 46]. The specific behaviors and phenotypes displayed by these mice can vary depending on the nature and location of the Shank3 mutation [45]. These

models offer insights into the role of synaptic proteins in ASD and have been instrumental in studying potential therapies targeting the Shank3 pathway.

2.1.3 PTEN Mutation Mouse Model

Phosphatase and tensin homolog (PTEN) is a critical tumor suppressor gene and phosphatase enzyme, whose primary function is to inhibit the phosphoinositide 3-kinase (PI3K) pathway [47, 48]. Thus, it plays a pivotal role in regulating cell growth, division, and death [47, 48]. While much research has been devoted to the involvement of PTEN in cancer development and progression, studies have also suggested a link between PTEN mutations and certain neurodevelopmental disorders, including ASD [49]. PTEN also plays an essential role in brain development and function, helping to regulate the growth, proliferation, and connectivity of neurons [48]. Disruption of the PI3K-AKT-mTOR pathway in the brain caused by PTEN mutations can affect synaptic plasticity, neurotransmission, and neuronal connectivity, potentially contributing to the development of ASD symptoms [49]. Recent studies of idiopathic ASD patients have detected a correlation between mTOR (mammalian target of rapamycin) pathway hyperactivation and clinical severity [50, 51].

Mice with PTEN mutations display increased brain size, social interaction deficits, and repetitive behaviors [37]. In the mouse model, the loss of PTEN function can lead to an overactivation of the PI3K-AKT-mTOR signaling pathway, which can affect neuronal development and connectivity [37]. Studying PTEN mouse models helps elucidate the relationship between brain overgrowth and ASD and offers a potential avenue for intervention targeting the PTEN pathway.

2.1.4 Neuroligin and Neurexin Mutant Mouse Models

Neuroligins (NLGNs) and neurexins (NRXNs) are cell adhesion molecules crucial for synapse formation and neuronal communication [52]. Mutations in these genes have been implicated in various neurological and neurodevelopmental conditions, including ASD [52]. There are multiple NLGN and NRXN genes, each encoding different isoforms of these proteins, allowing for a high degree of synaptic diversity [52]. The mutations in these genes can disrupt the balance of excitatory and inhibitory synapses, alter the function of glutamatergic and GABAergic signaling, and affect the development of neural circuits [52].

Mouse models with mutations in NLGN or NRXN genes exhibit altered synaptic structure and function, leading to impaired social interactions and repetitive behaviors [37]. These models provide a unique opportunity to explore the synaptic basis of ASD.

2.1.5 TSC1 and TSC2 Mouse Models

Tuberous Sclerosis Complex (TSC) is a genetic disorder often comorbid with ASD that involves mutations in either the TSC1 or TSC2 gene [53]. TSC1 and TSC2 are tumor suppressor genes that encode the proteins hamartin and tuberin, respectively [53]. These proteins form a complex that regulates the mTOR signaling pathway, which regulates cell growth and proliferation [53]. Mutations in TSC1 or TSC2 lead to an overactivation of the mTOR pathway [53]. This dysregulation results in abnormal cell growth and proliferation, leading to the formation of benign tumors in various organs, including the brain, skin, heart, and kidneys [53].

The TSC1 and TSC2 mouse models highlight the role of the mTOR pathway in ASD pathogenesis and have been crucial in testing potential mTOR-targeted treatments

for ASD [35]. TSC1 and TSC2 mutant mice often exhibit phenotypic characteristics that mimic aspects of human TSC, including the development of brain lesions (cortical tubers), skin abnormalities, renal cysts, cardiac rhabdomyomas, and cognitive deficits [35]. Furthermore, the behavioral and cognitive deficits observed in TSC1 and TSC2 mutant mice, such as deficits in social interactions, repetitive behaviors, and learning and memory difficulties, can help in better understanding the role of the mTOR pathway and molecular mechanisms underlying autism-like behaviors [35].

2.2. Environmental Mouse Models of ASD

While genetic factors play a significant role in ASD, there is mounting evidence to suggest that environmental factors also contribute to its etiology. These include prenatal factors such as maternal infections, toxicants, and nutritional deficiencies, as well as postnatal factors like pollutants, infections, and stress. To study the impact of these factors, researchers have developed prenatal, postnatal, and gene-environment interaction mouse models that mimic these environmental exposures.

2.2.1. Maternal Immune Activation (MIA) Models

Epidemiological studies have suggested that maternal immune responses to infections, immune challenges, or inflammation during pregnancy may impact fetal brain development and contribute to the risk of ASD in the child [54, 55]. Various maternal infections, including viral, bacterial, and protozoan infections, have been found to raise the risk of neurological and neuropsychiatric disorders in their children [54, 55]. In addition, the presence of chronic inflammatory conditions during pregnancy has been noted as a risk for offspring with neurodevelopmental disorders including ASD [54, 55].

It is hypothesized that MIA can lead to an increase in various pro-inflammatory molecules and cytokines in the maternal bloodstream [54, 55]. These immune molecules can potentially cross the placenta, entering the fetal circulation and activating immune cells [54, 55]. This leads to increased proinflammatory cytokine production, including IL-6 [54, 55]. Cytokines that cross the blood-brain barrier (BBB) can initiate neuroinflammation by activating microglia, triggering oxidative stress and mitochondrial dysfunction, creating a self-sustaining cycle that can harm brain development and behavior [54, 55].

MIA mouse models involve exposing pregnant mice to immune-stimulating agents, such as viral or bacterial mimetics, cytokines, or other immune system activators [46]. Studies using MIA models have shown that maternal immune activation can lead to changes in fetal brain development, including altered neural connectivity, abnormal behavior, and changes in gene expression [46]. Offspring born in these animal models often exhibit behavioral characteristics and deficits that are reminiscent of some features of ASD, such as social deficits, repetitive behaviors, and communication impairments [46].

2.2.2. Valproic Acid (VPA) Models

Prenatal exposure to valproic acid (VPA), an anticonvulsant and mood stabilizer medication often prescribed for epilepsy and bipolar disorder, has been associated with an increased risk of ASD and other neurodevelopmental disorders [56]. This link was first noted in observational research and has been supported by subsequent epidemiological and clinical investigations [56]. The timing of VPA exposure during pregnancy may be crucial, as research suggests that exposure during the first trimester, a

critical period for early fetal brain development, is associated with a higher risk of ASD [56]. It is thought that exposure can affect gene expression and epigenetic modifications, potentially contributing to the observed developmental and behavioral issues in the offspring. However, the exact mechanisms remain a subject of ongoing research [56].

Mice prenatally exposed to VPA exhibit social deficits and repetitive behaviors similar to those seen in clinical ASD [46, 57]. Furthermore, preclinical studies have shown that VPA can disrupt various cellular and molecular processes in the developing brain, including neuronal migration, synaptic plasticity, and neurotransmitter regulation [57].

2.2.3. Folate Deficiency Models

Folate, also known as vitamin B9, is a crucial nutrient that plays a fundamental role in DNA synthesis, repair, and methylation processes [58]. It is essential for proper neural tube development during early pregnancy and for the health of the CNS [58]. Several studies have suggested a potential link between maternal folate deficiency during pregnancy and an increased risk of autism in offspring [58]. These studies have primarily focused on the role of folate in epigenetic modifications, DNA methylation, and gene expression regulation during fetal brain development [58]. Folate deficiency may disrupt these processes, potentially leading to changes in gene expression patterns associated with autism [58]. Large-scale population-based studies have found a significant association between maternal use of prenatal vitamins containing folic acid during the first month of pregnancy and a reduced risk of autism in the offspring [59, 60]. This finding underscores the potential protective role of folate supplementation during early pregnancy.

In rodent models, researchers can induce maternal folate deficiency during pregnancy through dietary restrictions or genetic manipulation [61, 62]. Offspring in folate deficiency models demonstrate ASD-related behavioral changes including sociability deficits, as well as molecular alterations in DNA methylation patterns, histone modifications, and gene expression profiles [62]. Furthermore, dietary administration of folic acid in genetic folate deficiency models has been shown to reduce risk of ASD-like behavior in offspring [62].

2.2.4. Environmental Toxicant Models

Exposure to environmental toxicants can be harmful to human health and can occur through various routes, such as air, water, food, and occupational or household sources. These toxicants include heavy metals (e.g. lead and mercury), air pollutants, pesticides, endocrine-disrupting chemicals, and other pollutants. Several studies have suggested a potential link between prenatal and early-life exposure to environmental toxicants and an increased risk of autism in children.

Block et al. (2022) found that prenatal exposure to environmental air pollutants and subsequent MIA led to impaired microglial function during development, alterations in neural circuit formation and sustained behavioral abnormalities into adulthood [63]. Additional studies using mouse models in which pregnant mice are exposed to air pollutants found that offspring exhibit neuroinflammation, oxidative stress, and ASD-like behaviors [64].

2.2.5. Gene-Environment Interaction Models

Incorporating genetic mutations associated with ASD into environmental mouse models provides insight into gene-environment interactions [65]. Examples include

models with mutations in genes like MeCP2 or Shank3, combined with prenatal or postnatal environmental exposures.

2.3. Idiopathic Mouse Models of ASD

While researchers have identified various genetic and environmental factors that can contribute to the development of autism in some individuals, for the majority of cases, the exact cause remains elusive. "Idiopathic autism" is used to distinguish cases of autism where no specific genetic, environmental, or other known factors can be identified as the cause.

2.3.1. BTBR Mouse Model

BTBR T+Itpr3tf/J (BTBR) mice are an inbred mouse strain carrying the mutations a^{t} (nonagouti; black and tan), $Itpr3^{tf}$ (inositol 1,4,5-triphosphate receptor 3; tufted), and *T* (brachyury) (http://jaxmice.jax.org/strain/002282.html). BTBR mice have become an established model of idiopathic ASD, in part due to their strong behavioral face validity [66, 67]. As compared to C57BL6/J (B6) mice controls, BTBR mice exhibit reduced social interaction, increased repetitive self-grooming, unusual pattern of ultrasonic vocalizations and high anxiety [66, 67].

Furthermore, BTBR mice are known to possess ASD-associated genetic mutations and neuroanatomical changes that have been implicated in clinical ASD [68]. Transcriptomic and proteomic analysis of BTBR mice have demonstrated genetic alterations in pathways related to synaptic transmission, neurogenesis, axon guidance, regulation of actin cytoskeleton and immune regulation [68]. Their neuroanatomical aberrations, such as the absence of a corpus collosum and reduced hippocampal

commissure [69], mimic those seen in postmortem brain tissue samples [70, 71]. Researchers have noted changes in the shape and size of various BTBR brain regions, including the amygdala and hippocampus, that are similar to anomalies seen in certain subpopulations of ASD patients [68].

The aberrant immune profile observed in BTBR mice is also translationally relevant and includes alterations in immune cell populations, inflammatory response, and neuroimmunomodulation [68, 72]. Reports have shown that both peripheral and central immune cell populations are significantly upregulated in this inbred strain, as compared to B6 mice. Specifically, Heo et al. demonstrated an increase in $CD4^+$ T cells and $CD8^+$ T cells in the peripheral organs (including the spleen, blood and mesenteric lymph node) and an increase in MHC class II-expressing microglial cells in brains isolated from BTBR mice [73]. Multiple labs have also shown an increased production of proinflammatory cytokines (including IL-33, IL-18, and IL-1β) in the whole brain and specific brain regions (particularly the substantia nigra and cerebellum) of BTBR mice [73]. Similar to patient studies, there is evidence of Th1/Th2 imbalance, as measured by cell-specific cytokines, in BTBR mice that correlates with autistic trait severity. BTBR experiments have demonstrated a correlation between elevated IL-33 cytokine levels and more impaired autistic-like behavior [73]. In all, BTBR mice display a complex genetic, physiological, and behavioral background that has significant translational relevance.

2.4 Limitations of Mouse Models of ASD

While mouse models have proven invaluable in advancing our understanding of ASD and identifying potential therapeutic interventions, they are not without limitations.

One of the primary concerns of using mouse models for ASD research is the inherent difference between animal species and humans. While animals, especially rodents like mice and rats, share some genetic and physiological similarities with humans, they are still fundamentally different in terms of brain structure, cognitive abilities, and social behaviors [74]. Mice have smaller brains with distinct regions and connectivity patterns, which may not accurately model the complexity of the human brain. This disparity is particularly problematic when studying higher-order cognitive functions and complex social behaviors that are central to ASD [75].

Furthermore, ASD is a highly heterogeneous disorder that results from a wide range of genetic, environmental, and epigenetic factors. Animal models often focus on specific genetic mutations or environmental exposures, which may not fully capture the diverse causes of ASD in humans [76]. Consequently, findings from animal models might not be relevant to individuals with different genetic or environmental backgrounds, limiting the generalizability of research results. The heterogeneity of the disorder means that no single mouse model can fully represent all aspects of ASD. Researchers often need to use multiple models to address the diversity of genetic and neurobiological underpinnings seen in ASD.

Animal models have provided crucial insights into potential mechanisms and therapeutic approaches. However, it is essential to recognize their limitations when conducting translational research. The inherent differences between animal species and humans, imprecise behavioral measures, and limited cognitive abilities, and etiological heterogeneity all pose significant challenges to accurately modeling ASD in animals and translating these findings to the human context. While animal models remain essential for

some aspects of autism research, researchers must be cautious about overgeneralizing preclinical findings. A more comprehensive approach that combines data from animal models with human studies, including genetics, neuroimaging, and clinical observations, is necessary to overcome these limitations and advance our understanding of ASD for the development of effective treatments.

III. Cannabinoids and The Endocannabinoid System

3.1. A Brief History of Cannabinoid Use and Research

The history of cannabinoids is a fascinating journey that spans thousands of years and involves various cultures, scientific discoveries, legal regulations, and changing perspectives. The use of cannabis plants for medicinal and recreational purposes can be traced back thousands of years. Ancient Chinese texts from around 2737 BC describe the medicinal properties of cannabis, while the sacred Hindu text, the Atharvaveda, mentions the plant's psychoactive effects and use in ancient India for religious purposes [77]. As trade routes and exploration expanded, cannabis found its way to the Middle East, Africa, and Europe [77]. The 19th century marked a turning point in the study of cannabinoids. In 1839, the Irish physician William O'Shaughnessy introduced cannabis to Western medicine, highlighting its potential as an analgesic, antiemetic, and anticonvulsant [78]. This led to a surge of interest in cannabis-based medicines and the development of various cannabis tinctures and extracts, paving the way for further research into cannabis and its compounds [78].

The first cannabinoid, cannabidiol (CBD), was isolated in 1940 [78]. Subsequently, the psychoactive cannabinoid, delta-9-tetrahydrocannabinol (THC), was

identified and synthesized by Dr. Raphael Mechoulam in the 1960s [79]. However, during this time there was a growing concern about the recreational use of cannabis, leading to the criminalization of marijuana in many countries. The U.S. Controlled Substances Act of 1970 classified cannabis as a Schedule I drug, along with heroin and LSD [80]. Despite the strict regulations that severely restricted research, scientists continued to investigate the potential medical benefits of cannabis. Research into THC's mechanism of action led to the discovery of the Endocannabinoid System in the 1990s and led to a better understanding of the pharmacological effects of cannabinoids [78].

Today, cannabis and its cannabinoid constituents are purported to have a wide range of medical applications include the treatment of multiple sclerosis, chronic pain, cancer [81], inflammation [82], epilepsy [83, 84], neurodegenerative disorders [85, 86], and psychiatric diseases [87]. Medical cannabis legalization in various states and countries in recent years reflects a shift towards recognizing the therapeutic value of cannabis. In recent years, some regions have also moved toward the legalization of recreational cannabis use, acknowledging the cultural and historical significance of this plant and the desire to regulate its use in a responsible manner. As societal attitudes continue to evolve, it is likely that the role of cannabinoids in medicine and society will also continue to change.

3.2 The Endocannabinoid System

The Endocannabinoid System (ECS), comprised of the cannabinoid receptors 1 (CB1) and 2 (CB2), their endogenous ligands (ie. endocannabinoids) and corresponding biosynthetic and metabolizing enzymes, plays a key role in the regulation of a myriad of

essential neuronal and immune processes [88]. This neuro-modulatory system has been implicated numerous neurological and neurodevelopmental pathologies, and pharmacological interventions that target the ECS have shown therapeutic promise (Parrella).

3.2.1 Cannabinoid Receptors

CB1 and CB2, first cloned in 1990 and 1993 respectively, are rhodopsin-like, class A metabotropic G protein-coupled receptors (GPCRs) expressed both centrally and peripherally [89, 90]. CB1 is expressed predominantly in presynaptic neurons of the CNS, particularly the cerebral cortex, hippocampus, basal ganglia, and cerebellum, and to a lesser extent in the PNS [89-93]. In fact, in 1991, Herkenham et al. demonstrated CB1 to be the most abundant GPCR in the mammalian brain [91]. Contrastingly, CB2 is found largely in immune cells, with only minor expression in neurons [90, 94-98]. More recently, both CB1 and CB2 have been reported in other peripheral tissues, including pancreatic β-cells, osteoblasts, osteocytes, and osteoclasts [99-101]. The cannabinoid receptors signal through a complex array of pathways (Figure 3). Primarily coupled to $G_{i/o}$ proteins, these receptors situated on the presynaptic neurons can regulate the inhibition of adenyl cyclase activity and the activation of the MAPK cascade [89, 98, 102, 103]. They can also stimulate K^+ channels [104, 105] and inhibit voltage-gated Ca^{2+} channels [104, 106, 107]. In addition, CB1 has been found activate PLC and cause calcium mobilization via G_q coupling [108].

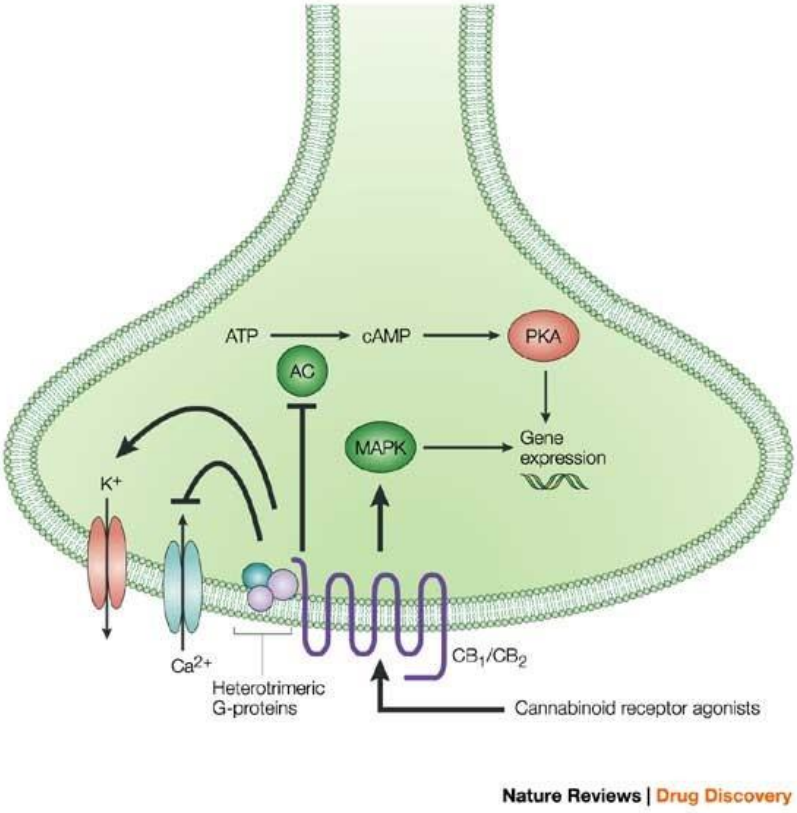

Figure 3. CB1 and CB2 signaling pathways activated by cannabinoid receptor agonists

Cannabinoid receptor agonists can bind to and activate CB1 and CB2. These receptors, primarily coupled to $G_{i/o}$ heterotrimeric proteins, can then inhibit adenylate cyclase (AC) activity, thereby reducing cyclic AMP (cAMP), as well as activate Mitogen-Activated Protein Kinase (MAPK). These two signaling cascades contribute to regulation of gene expression. CB1 and CB2 agonism can also lead to the inhibition of voltage-gated Ca^{2+} channels and the stimulation of inwardly rectifying K^+ channels. This figure is adapted from Di Marzo et al. (2004) [109].

3.2.2 Endocannabinoids & Regulating Enzymes

Endocannabinoid ligands are endogenous (naturally occurring) compounds produced by the body that bind to and activate the cannabinoid receptors CB1 and CB2. Anandamide (AEA) and 2-arachidonylglycerol (2-AG) were the first of the endocannabinoids to be discovered (Figure 4) [110-112]. While these two lipid mediators are considered the archetypal endocannabinoids, researchers have since identified additional cannabimimetic compounds, including the N-acylethanolamines and N-acyl dopamines [113].

AEA was named after the Sanskrit word "ananda," meaning "bliss" or "joy," due to its association with feelings of well-being. It is synthesized on-demand from arachidonic acid and other cell membrane precursors, usually in response to physiological changes or stress [114]. It primarily binds to and activates CB1 receptors found in the CNS, but also has a lower affinity for CB2 receptors located primarily in the PNS and immune cells [114]. AEA is involved in pain regulation, mood, appetite, and neuroprotection [114]. It also plays a role in the formation of short-term memories and contributes to the analgesic effects of exercise, often referred to as the "runner's high" [114].

Like AEA, 2-AG is produced on-demand in response to cellular and environmental cues [114]. It primarily binds to and activates CB1 receptors, especially in the brain, where it is involved in the modulation of neurotransmitter release [114]. 2-AG has been linked to pain perception, inflammation, and appetite regulation [114].

AEA and 2-AG are synthesized by N-acylphosphatidylethanolamine-specific phospholipase D-like hydrolase (NAPE-PLD) and diacylglycerol lipase α and β (DAGLα

and DAGLβ), respectively. Enzymes like fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) are responsible for breaking down these and other endocannabinoids after they have fulfilled their function. Regulation by these biosynthetic and metabolizing enzymes prevents the excessive activation of the ECS.

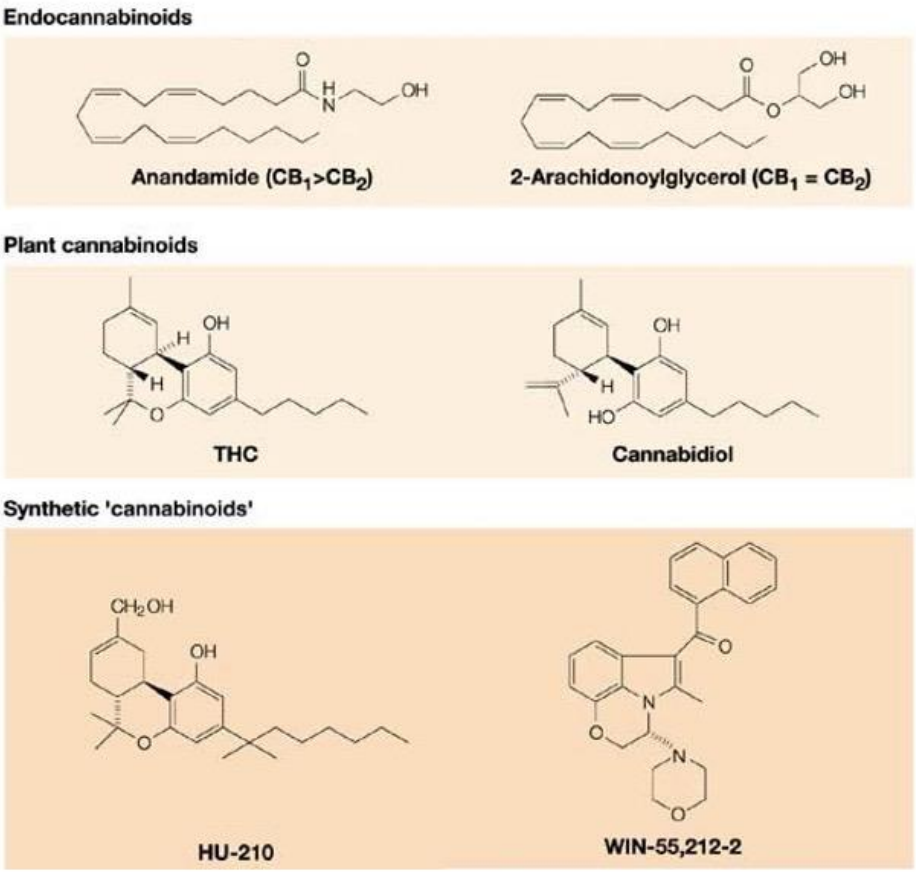

Figure 4. Chemical structures of major endocannabinoids, phytocannabinoids and synthetic cannabinoids

Endocannabinoids: Anandamide binds with greater affinity to CB1 than CB2, while 2-Arachidonylglycerol binds to CB1 and CB2 with equal affinity. Phytocannabinoids: THC is the major psychoactive constituent of Cannabis sativa, while cannabidiol is the major non-psychoactive constituent. Synthetic cannabinoids: HU-210 and WIN-55,2-2-2 are potent cannabimimetic agonists. This figure is adapted from Di Marzo et al. (2004) [109].

3.2.3 Phytocannabinoids & Synthetic Cannabinoids

Exogenous ligands, such as phytocannabinoids and synthetic cannabinoids (Figure 4), elicit cannabimimetic effects by acting on the CB1 and CB2 receptors, thereby modulating the ECS. Phytocannabinoids are plant-derived exogenous ligands that have garnered much attention in recent years for its purported therapeutic properties. The most studied source of these naturally occurring compounds is *Cannabis sativa*, containing over 65 known cannabinoids, to date [115]. Its various medical applications include the treatment of multiple sclerosis, chronic pain, cancer [81], inflammation [82], epilepsy [83, 84], neurodegenerative disorders [85, 86], and psychiatric diseases [87]. Although Δ^9-tetrahydrocannabinol (THC) is the principal component of *Cannabis sativa*, its psychoactive properties limit its therapeutic uses. By contrast, the major non-psychoactive constituents, cannabidiol (CBD) and cannabidavarin (CBDV), offer pharmacological advantages over THC as they display lower toxicity/fewer adverse effects in patients [116, 117]. Currently, it is unknown how these drugs elicit their therapeutic responses; both their molecular targets and polypharmacological mechanisms of action remain poorly understood.

Synthetic cannabinoids are a structurally diverse class of compounds designed for research and therapeutic purposes [118]. Most of these compounds are CB1 and CB2 agonists, displaying higher affinity and potency for these receptors compared to THC or CBD [119]. A number of these compounds, including WIN55,212-2 and HU-210, have shown therapeutic benefits in animal models, such as analgesic, anticonvulsant, and anti-inflammatory effects [118]. However, recreational abuse of some synthetic cannabinoids

has been associated with a range of adverse health effects, including cardiovascular issues, respiratory problems, seizures, and mental health disturbances [118, 119].

3.3 The Physiological Role of the ECS:

The ECS plays a role in regulating various physiological processes, including:

Pain Perception: The ECS is involved in the modulation of pain perception, and it plays a role in the analgesic effects of cannabinoids [114]. Endocannabinoids, such as AEA and 2-AG, are produced in response to pain or injury, binding primarily to the CB1 receptors and in effect reducing the perception of pain [114].

Inflammation: The ECS can help modulate immune responses, including inflammation [120]. Activation of CB2 receptors can lead to a reduction in immune cell activation and the release of pro-inflammatory cytokines, along with an increase in the production of anti-inflammatory cytokines [120]. Targeting the ECS has been explored in the development of anti-inflammatory therapies for conditions like arthritis and inflammatory bowel disease [120]. The ECS also plays a role in regulating neuroinflammation in the central nervous system [113]. This is significant in the context of neurodegenerative diseases, as excessive inflammation in the brain can contribute to the progression of conditions like Alzheimer's and Parkinson's disease [113].

Appetite and Metabolism: The ECS is involved in the regulation of appetite, food intake, and energy balance [120]. Activation of the CB1 receptor in the hypothalamus and other brain regions associated with feeding behavior can stimulate appetite, and an increase in AEA levels, can lead to an increase in food intake [120]. The ECS also plays a role in metabolic functions, and can influence how the body processes and stores

energy [120]. Activation of CB1 receptors in peripheral tissues, such as adipose tissue and the liver, can affect lipid metabolism and insulin sensitivity [120].

Mood and Emotions: The ECS is associated with mood regulation, and alterations in its function have been linked to conditions like anxiety, depression, and stress [113]. Activation of the ECS, particularly through the CB1 receptors in the brain, can have anxiolytic effects [113]. Endocannabinoids and certain phytocannabinoids can bind to these receptors and dampen the perception of anxiety and stress [113].

Neuroprotection: It is believed that the ECS plays a role in protecting and maintaining the health of nerve cells, which has implications for neurodegenerative and neurodevelopmental conditions [113]. The ECS helps regulate the balance of neurotransmitters in the brain, including glutamate, a major excitatory neurotransmitter [113]. Excessive release of glutamate can lead to excitotoxicity, a process that contributes to nerve cell damage and death in conditions such as stroke and neurodegenerative diseases [113]. The ECS can modulate glutamate release, potentially protecting neurons from excitotoxic damage [113]. Furthermore, activation of cannabinoid receptors, such as CB1, has been associated with increased neurogenesis in certain brain regions [113].

Memory and Learning: The ECS influences memory and learning processes, and its modulation has been explored in the context of cognitive disorders [113]. The hippocampus, a region of the brain critical for memory formation and spatial navigation, contains a high density of CB1 receptors [113]. Endocannabinoids, such as AEA, are modulate synaptic plasticity and memory processes in the hippocampus [113].

Reproductive Processes and Fertility: The ECS is involved in the regulation of both male and female reproductive processes and fertility [114]. In female individuals,

the ECS plays a role in ovulation, embryonic implantation, uterine contracts, and the menstrual cycle [114]. Imbalances in endocannabinoid signaling can lead to disruptions in ovulation, irregular menstrual cycles and, in some cases, conditions like polycystic ovary syndrome (PCOS) [114]. In males, the ECS is involved in the regulation of sperm production, sperm motility, ejaculation and erectile function [114].

IV. Cannabidiol and Autism Spectrum Disorder

Cannabidiol (CBD), the principal non-psychoactive constituent of *Cannabis sativa*, was first isolated and identified by Dr. Roger Adams and his team at the University of Illinois in 1940 from a sample of Minnesota wild hemp [121]. However, initial research on the phytocannabinoid was relatively limited and often overshadowed by interest in the psychoactive effects of THC [122]. As attitudes toward cannabis and its compounds began to evolve in the late 20th century, renewed interest in CBD eventually led to the exploration of its diverse potential therapeutic applications. To date, CBD has been demonstrated to exhibit neuroprotective [86, 123, 124], anti-inflammatory [122, 123, 125, 126], anti-convulsant [127-129], anxiolytic [130, 131], anti-psychotic [132, 133], and anti-nausea properties [134]. In 2018, CBD (Epidiolex) became the first FDA-approved cannabis-derived drug. It is currently indicated for the treatment of seizures involved in Lennox-Gastaut Syndrome, Dravet Syndrome, and Tuberous Sclerosis Complex (TSC) [135]. While much still needs to be elucidated, emerging evidence suggests CBD's therapeutic benefit in other neurological and neurodevelopmental pathologies as well, including ASD [136].

4.1 CBD and Preclinical Models of ASD

Preclinical mouse models of ASD have provided valuable insights into the potential therapeutic effects of various pharmacological agents on the neurobiological underpinnings of the disorder and on ASD-related behaviors. While CBD has shown efficacy in various neurological and psychiatric conditions [137], its potential benefits in ASD are still under investigation.

Much of the preclinical evidence for CBD's application in ASD is merely suggestive, based on findings in a mouse model of Dravet Syndrome (DS), a rare and severe form of epilepsy that typically begins in early infancy. Formerly known as Severe Myoclonic Epilepsy of Infancy (SMEI), DS is primarily caused by mutations in the *SCN1A* gene, which encodes sodium voltage-gated channels, and is characterized by frequent and prolonged seizures, developmental delays, and cognitive impairments [138]. DS patients can exhibit a myriad of behavioral problems ranging from irritability and hyperactivity to narrow interests and social withdrawal [138, 139]. Reports have suggested that more than 50% of DS patients display autistic features and ~25-60% are co-diagnosed with ASD [138-141].

The *Scn1a* $^{+/-}$ mouse model of DS demonstrates not only clinically relevant seizures [142], but behavioral alterations as well, including low sociability, poor spatial learning ability, hyperactivity, anxiety-like behavior, and increased stereotypies [143, 144]. Kaplan et al. (2017) investigated the effects of CBD on both the epileptic and behavioral phenotypes in this genetic model of DS. CBD was able to attenuate seizures at high doses (100 mg/kg and 200 mg/kg) in male and female juvenile *Scn1a* $^{+/-}$ mice, reducing frequency, duration and severity [142]. Furthermore, they found that acute i.p.

administration of low doses of CBD (10 mg/kg or 20 mg/kg) in adult male *Scn1a* $^{+/-}$ mice increased sociability in the three-chamber social interaction test [142]. Notably, this beneficial effect was not observed following 50 mg/kg CBD treatment [142]. In the open field test, they noted that acute 100 mg/kg CDB dosing reduced hyperactivity in the heterozygote mice, with distance traveled levels comparable to WT mice, but did not impair locomotor activity, as determined by velocity of movement [142].

In a subsequent study, Patra et al. (2020) examined the effects of chronic CBD treatment in this same mouse model of DS [145]. *Scn1a* $^{+/-}$ mice were subcutaneously injected twice daily with either vehicle or 100 mg/kg CBD from postnatal day (pnd) 8 to pnd52/death [145]. Home cage social interaction tests performed between pnd35 and pnd37 revealed that chronic CBD administration significantly improved social behavior [145]. Anxiety-like behavior measured by the elevated plus maze on pnd 42 was also attenuated in the CBD-treated mice. CBD did not adversely affect motor function or gait in the accelerating rotarod and static beam tests [145].

A very recently published study analyzed the effects of acute CBD treatment on behavior in the BTBR mouse model [146]. Adult (4-6 month old) male BTBR mice were injected (i.p.) with vehicle, 0.1, 1, or 10 mg/kg CBD 30 minutes prior to undergoing a battery of behavioral tests that assessed social interaction preference, social novelty preference, marble burying and social dominance [146]. Ferreira et al. (2023) found that BTBR mice treated with 10 mg/kg CBD, but not 0.1 or 1 mg/kg CBD, exhibited increased social interaction compared to vehicle-treated controls. In the social preference, marble burying or social dominance tests, however, CBD did not have a significant behavioral effect at any acute dose (ie. 0.1-10 mg/kg CBD) in the BTBR mice.

Taken together, the CBD-induced behavioral improvements observed in the both the *Scn1a* $^{+/-}$ and BTBR mouse models suggest that CBD may hold promise as a treatment option for ASD. However, translating these findings to clinical applications requires further investigation into the mechanisms of action, long-term safety, and optimal dosing.

4.2 CBD and Clinical ASD

Clinical trials testing CBD for the treatment of ASD symptoms are limited, with many still in the recruitment phase (clinicaltrials.gov). Moreover, a large portion of the completed studies involved administration of THC/CBD formulations, rather than formulations containing purified CBD alone . Nevertheless, the preliminary clinical findings in autistic patients show promise for CBD as a therapeutic intervention.

In one of the first observational studies, Barchel et al. (2018) investigated the use of oral CBD-enriched cannabis extracts in 53 children with ASD and overall outcome as assessed by participant's parents [147]. Participants (85% male), who ranged in age from 4 to 22 years old with a median age of 11, received a cannabinoid oil containing a 20:1 CBD/THC ratio for a median duration of 66 days (30-588) [147]. Analysis of data reported by parents indicated significant improvements in self-injury and rage attacks in 67% of patients, hyperactivity symptoms in 68.4% of patients, and anxiety in 47.1% of patients [147]. Adverse events were all noted to be mild, with the most common being somnolence and changes in appetite [147]. While this study did have several limitations, including subjective reporting and a lack of control group, it does suggest that CBD may be useful in treating ASD-related co-morbid symptoms such as anxiety and hyperactivity.

Pretzsch et al. (2019) investigated the effects of CBD on excitatory and inhibitory neurotransmitter levels in the brains of both neurotypical (N = 17) and autistic (N = 17) males, using magnetic resonance spectroscopy (MRS) [148]. This placebo-controlled, randomized, double-blind, repeated-measures, cross-over study was conducted as part of a larger clinical trial investigating phytocannabinoids and ASD (NCT03537950) [148]. The mean age for typically developing (TD) individuals was 28.47 $\pm$ 6.55 years, while the mean age of participants with ASD was 31.29 $\pm$ 9.94 years [148]. Potential participants were excluded if they had a genetic disorder associated with ASD, such as TSC or FXS, or an IQ below 70 [148]. Participants received a single oral dose of either 600 mg CBD or placebo, with imaging data acquisition beginning 2 hours post-drug administration to coincide with peak plasma concentrations [148]. MRS data showed that in both the TD and ASD groups following CBD treatment, levels of glutamate were increased in the basal ganglia and decreased in the dorsomedial prefrontal cortex [148]. However, when it came to GABA+ levels, the response to CBD differed between the two groups [148]. Specifically, in both prefrontal and subcortical regions, CBD increased GABA+ levels in individuals without ASD but decreased levels in those with ASD [148]. They concluded that while the excitatory glutamate response to CBD remains similar across both groups, the inhibitory GABA response pathways appear to be altered in individuals with ASD [148]. Together, their findings suggest that CBD may have a distinct functional impact on the inhibitory GABA response pathways in individuals with ASD [148].

Using this same cohort of TD and ASD patients and in conjunction with their larger investigation (NCT03537950), Pretzsch et al. (2019) conducted another study to

measure brain responsivity to CBD using the resting state functional magnetic resonance imaging (fMRI) [149]. In both groups, CBD significantly increased the fractional amplitude of low-frequency fluctuations (fALFF), an indicator of spontaneous regional brain activity, in the cerebellar vermis and the right fusiform gyrus [149]. Post-hoc within-group analysis demonstrated that this drug effect was significant in the ASD group, but not in the TD group [149]. They subsequently measured functional connectivity (FC) in the two brain regions where CBD significantly altered fALFF [149]. CBD induced significant changes in the vermal FC within the ASD group exclusively, but did not affect fusiform FC in either group [149]. Abnormalities in both the cerebellum and the right fusiform gyrus have been implicated in ASD. This makes sense as the former is associated with motor skills, language and emotional control, while the latter is involved in visual processing of faces and words. The results of this study suggest that CBD-induced alterations of regional fALFF and associated FC could have functional implications on behavior and cognitive processes in autistic patients.

Aran et al. (2019) performed a retrospective feasibility study assessing 60 children with ASD and severe behavioral problems [150]. The patient group was composed of 83% boys, with an age range of 5 to 17.5 years old and a mean age of 11.8 ± 3.5 years; 77% of participants were considered low function based on prior psychological evaluations using the Autism Diagnostic Observation Schedule (ADOS) or Childhood Autism Rating Scale (CARS) [150]. All patients had a score of 6 or 7 on the Clinical Global Impression Scale—Severity (CGI-S), indicating severe behavioral problems [150]. Participants were given cannabidiol-rich cannabis whole plant extract containing a CBD/THC ratio or 20:1 or 6:1 [150]. A portion of patients experienced

adverse events including sleep disturbances (17%), irritability (9%), and loss of appetite (9%) [150]. Based on the Caregiver Global Impression of Change (CGIC) scale, behavioral problems were 'much improved' or 'very much improved' in 61% of children [150]. Furthermore, anxiety and communication problems improved in 39% and 47% of patients respectively [150].

Ultimately these promising findings led Aran et al. (2021) to conduct a placebo-controlled double-blind trial (NCT02956226) in 150 children and adolescents with ASD [151]. Participants, which were 80% male, ranged in age from 5 to 21 years old, with a mean age of 11.8 ± 4.1 years, and exhibited moderate or greater behavioral problems (rating ≥ 4) based on the Clinical Global Impression Scale—Severity (CGI-S) scale [151]. Participants were randomly assigned (1:1:1 ratio) to 1 of 3 treatments: oral placebo, whole-plant cannabis extract containing 20:1 CBD/THC ratio (BOL-DP-O-01-W), or purified CBD and THC at the same ratio and concentration (BOL-DP-O-01) [151]. The treatments were administered orally in three daily doses over a span of 12 weeks. This was followed by a 4-week washout period, after which a predetermined cross-over took place for an additional 12 weeks to further evaluate tolerability [151]. Aran et al. (2021) noted that they chose not to include a group solely administered with CBD, as they hypothesized that the combination of CBD and THC would be more effective due to THC's direct impact on the endocannabinoid system [151]. While no treatment-related severe or serious adverse events were observed, some mild to moderate adverse events included somnolence, decreased appetite, weight loss, tiredness, euphoria and anxiety [151].

The effect of cannabinoid treatment on behavioral issues was evaluated using the Home Situations Questionnaire–Autism Spectrum Disorder (HSQ-ASD) and Clinical Global Impression-Improvement (CGI-I) as co-primary outcome measures. The child's behavior was also assessed by the Autism Parenting Stress Index (APSI), which served as a secondary outcome measure [151]. When comparing participants who received cannabinoids with those who received a placebo, there were no significant differences in HSQ-ASD or APSI total scores [151]. However, the findings from the CGI-I assessment revealed that 49% of the 45 participants administered whole-plant cannabinoids demonstrated a positive response (ie. much or very much improved), in contrast to the 21% of the 47 participants who received a placebo ($p = 0.005$) [151]. In the case of the 45 participants given pure cannabinoids, 38% displayed a positive response on the CGI-I scale, although this difference was not statistically significant compared to the placebo group ($p = 0.08$) [151]. Notably, there did not appear to be a clear advantage of whole plant extract treatment over pure cannabinoids, as none of the three behavioral measures (HSQ-ASD, CGI-I, and APSI) differed significantly between the two groups [151]. Core ASD symptoms were assessed as a secondary outcome measure with the Social Responsiveness Scale (SRS-2). There was a significant improvement in the SRS-2 total score after whole-plant extract treatment compared to the placebo ($p = 0.009$) [151]. While SRS-2 total scores did improve following pure cannabinoid treatment, this effect did not differ significantly from the placebo group ($p = 0.80$) [151].

Collectively, these promising preliminary clinical studies suggest that CBD treatment could be effective in ameliorating core ASD symptoms and warrants further

investigation as a possible intervention in ASD. More extensive and rigorous clinical trials are needed to establish its safety, efficacy, dosing, and long-term effects.

CHAPTER II

CANNABIDIOL IS A BEHAVIORAL MODULATOR IN BTBR MOUSE MODEL OF IDIOPATHIC AUTISM

I. Introduction

Autism spectrum disorder (ASD) is a heterogenous group of neurodevelopmental disorders, clinically characterized by three core symptoms: impaired social interactions, social communication deficits, and repetitive behaviors with restricted interests [1]. With prevalence drastically rising over the last two decades to an estimated 1 in 36 children, ASD has become one of the most common neurodevelopmental disorders [4]. The molecular mechanisms underlying ASD pathogenesis remain elusive. The majority of cases are idiopathic, with only ~20-30% attributable to rare genetic variants including *de novo* mutations, copy number variants and monogenic disorders, such as Fragile X Syndrome, Tuberous Sclerosis Complex, and Rett Syndrome [11, 152, 153]. Based on twin and familial epidemiological studies, the etiology of ASD is thought to involve a complex interaction between diverse genetic, epigenetic and environmental risk factors [7-10].

Currently, no genetic, neuroimaging, or electrophysiological tests exists to definitively diagnose patients. Instead, ASD is clinically diagnosed by evaluation and characterization of behavioral, social and cognitive patterns, with a broad range and severity of symptoms unique to each patient [19, 20]. Onset of ASD typically occurs in early childhood, with most core symptoms appearing between 12 and 24 months of age

[18]. Physicians and researchers place an emphasis on the importance of early diagnosis, as considerable evidence supports the benefit of early intervention on long-term patient outcome [18, 23-25]. In addition, ASD is often accompanied by several comorbidities, including attention deficit hyperactivity disorder (ADHD) and anxiety disorder, which introduces complexity in making diagnoses and establishing individualized treatment plans.

There is a significant unmet need for the development of therapeutic interventions for ASD [154]. Standard of care at present relies heavily on behavioral therapy, which has varying success among this diverse patient population. To date, a pharmacological intervention for the treatment of core autistic symptoms has yet to be successfully developed. *Cannabis sativa* has garnered recent attention for its potential therapeutic applications and is demonstrated to have analgesic, antiemetic, anticonvulsant, neuroprotective, anti-inflammation and antitumor properties [115, 127, 155, 156]. In particular, cannabidiol (CBD), the primary nonpsychoactive component of *Cannabis*, has been approved by the US FDA for the treatment of seizures associated with Lennox-Gastaut syndrome, Dravet syndrome, and tuberous sclerosis complex [135]. Case reports and pilot clinical trials have suggested that the cannabinoid may also be effective in targeting core autistic symptoms [157]. However, the efficacy of purified CBD in treating idiopathic ASD has not been thoroughly investigated in preclinical or clinical studies.

BTBR $T^{+}Itpr3^{tf}$/J (BTBR) mice are an inbred mouse strain carrying the mutations a^{t} (nonagouti; black and tan), $Itpr3^{tf}$ (inositol 1,4,5-triphosphate receptor 3; tufted), and *T* (brachyury) (http://jaxmice.jax.org/strain/002282.html). BTBR mice have become an established model of idiopathic ASD, in part due to known ASD-associated genetic

mutations and their strong behavioral face validity [66-68]. As compared to C57BL6/J (B6) mice controls, BTBR mice exhibit reduced social interaction [67, 158-160], increased repetitive self-grooming [67, 161-163], and increased locomotor activity [67, 164-166]. The current study tested the hypothesis that CBD administered in the immediate post-weaning period can rescue both core behavioral deficits and autism-associated comorbid symptoms in a mouse model of idiopathic ASD. Behavioral assays were implemented to examine repetitive self-grooming, sociability, and locomotor activity in BTBR mice, following daily intraperitoneal (i.p.) treatment of vehicle or CBD for two weeks.

II. Materials and Methods

2.1. Materials

Cannabidiol was purchased from Cayman Chemical (Ann Arbor, MI). Ethanol and Tween 20 were purchased from Sigma Aldrich (St Louis, MO). 1 mL syringes and 27-gauge needles were purchased from VWR (Radnor, PA).

2.2. Animal Maintenance and Housing

This study was conducted according to a protocol approved by the University of Louisville Institutional Animal Care and Use Committee and NIH guidelines. Subjects were offspring of C57BL/6J (B6) and BTBR $T^{+}Itpr3^{tf}$/J (BTBR) breeding pairs obtained from Jackson Laboratory (Bar Harbor, ME). Animals were bred and housed in clean, federally regulated and AAALAC-accredited facilities operated by the University of Louisville School of Medicine Department of Animal Care. Subjects were male B6 and

BTBR mice between the ages of 3 weeks and 6 weeks of age. Mice were weaned at postnatal day (pnd) 21 and no more than five littermates were housed per cage (33 cm long x 19 cm wide x 15 cm high) with free access to food and water, in a humidity (30%) and temperature (22 ± 1°C) controlled room with a 12 h light/dark cycle (lights on at 0600 h). All animals utilized in this project were monitored daily for evidence of discomfort, distress, pain, or injury.

2.3. Animal Dosing

This study investigated the effects of CBD on the behaviors of male BTBR mice. CBD was dissolved in 5% ethanol, 5% Tween 20 and PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Weaned juvenile BTBR mice were administered daily via i.p. injection either vehicle, 20 mg/kg CBD or 50 mg/kg CBD treatment for two weeks, beginning at pnd21 ± 3 days. Following injection on the final treatment day (ie. pnd34 ± 3), mice were subject to a battery of behavioral testing as outlined below. Male age-matched vehicle-treated B6 mice were used as controls. N = 10-15 mice/dosing group.

2.4. Behavioral Room Set-up

All behavioral testing was conducted in an experimental room in the same building but separate from the animal care facilities. All experiments were conducted during the light cycle [167] between 0900 h and 1800 h. For each subject, the battery of behavioral assays was conducted within a single day. Following i.p. injection of drug or vehicle on final day of treatment, animals were brought in their home cages to the

experimental room to acclimate for 30 min to 1 h prior to testing. The room was kept quiet during the entire time animals were present. The order of testing was chosen in order to minimize stress impact on the animal, starting with the least stressful test and ending with the most stressful. Thus, the testing sequence began with the repetitive self-grooming assay, followed by the open field assay and concluding with the three-chamber sociability assay. Subjects were given a minimum of 45 min resting period in their home cage between each assay. Behavioral apparatuses were thoroughly cleaned with 70% ethanol and DI water between test subjects.

2.5. Machine Vision Analysis of Behavior

All behavioral recordings were carried out under red light in a quiet, dark, temperature-controlled room dedicated to this purpose. Recordings were carried out in a sound-proofed enclosure built using modular aluminum elements (McMaster-Carr, Aurora OH) that support experimental enclosures made of Plexiglas, to standard dimensions [167-169]. Image acquisition software has been developed in-house, using the LabView programming environment (National Instruments, Austin TX), running on a customized workstation (Windows 10, 32 GB RAM). Behavior was recorded by 2 cameras (Basler ace acA640-300gm GigE, Mono, 6mm UC Series Lens; Edmund Optics) providing top- and side-views of enclosures to simultaneously record ongoing behaviors (30 Hz). Behavior was analyzed using in-house software developed in LabView. Background subtraction and machine-vision utilities were used to track the mouse's movements. In-house software was benchmarked and validated against a commercially available package (Smart 3.0, Harvard Apparatus, Boston MA) with similar results. Total

path length, time spent in different parts of the enclosure, rearing and jumping were all automatically extracted. In addition, video segments corresponding to pauses in locomotion (based on minimum pause duration, and maximum movement during a pause) were extracted for subsequent inspection and manual scoring (grooming, social interactions, sniffing, object exploration). Fur color markings distinguished B6 mice (dark brown) from BTBR mice (dark brown with tan ventral patch), and prevented observers from being fully blind to strain during manual scoring of behaviors. All data were exported to excel (Microsoft, Redmond WA), and summary statistics for each experiment, and summaries of summaries were all generated using macros within Excel.

2.6. Repetitive self-grooming assay

The repetitive self-grooming assay used to measure restricted interest/repetitive behavior was carried out following a previously published protocol [170]. Briefly, a single mouse subject was placed in a clean, empty cage with 1 cm of bedding, and allowed to freely explore. The mouse's behavior and movements were recorded for 10 min. From the video recordings, cumulative time spent self-grooming was scored by the researcher using automated image capture. Time spent performing different behaviors (rearing, grooming, etc.) was manually scored by the observer using automated image capture. Spontaneous self-grooming behavior included paw/leg licking, head washing, genital/tail grooming and body grooming. Fresh, clean cages were used for each subject. Subjects were returned to their home cages following testing.

2.7. Open Field Assay

The open field assay was subsequently used to determine whether CBD had an effect on exploratory locomotor activity and anxiety-like behavior. The open field assay was conducted in a square white plexiglass chamber (60 cm long x 60 cm wide x 26 cm high) and followed a previously published protocol [171]. Individual subjects were placed in the center of the open field chamber and allowed to freely explore. Mouse movements and behavior were recorded for 10 min. Distance travelled was measured using the in house Machine Vision tracking system. Time spent in the center and edges of the chamber were recorded to assess anxiety-related behavior.

2.8. Three-Chamber Sociability Assay

The three-chamber assay evaluated the sociability phenotype of B6 and BTBR mice with or without CBD treatment following a published protocol [172]. The three-chamber apparatus was a rectangular Plexiglas box with each chamber measuring 48 cm long x 19 cm wide x 25.5 cm high. Plexiglass walls dividing the chambers contained a 18 cm opening to allow access between the chambers. Briefly, the subject mouse was given a 10 min habituation period in the middle chamber with side chambers blocked off, followed by a 10 min habituation period to the entire empty arena with free access to the center and side chambers. The subject mouse was then briefly confined to the center chamber. A socially unfamiliar, age- and sex-matched B6 mouse (novel mouse) was placed in an inverted wire cup in the center of one the side chambers, while an empty inverted wire cup (novel object) was placed in the center of other side chamber. An upright beaker was placed on top of each inverted wire cup to prevent the subject from

climbing onto the top of the wire cups. The side doors were then opened, the subject mouse was given free access to the entire arena and its movements were recorded for 10 min. The side chamber placement of the novel mouse and novel object was alternated between test mice, and a lack of innate side preference was confirmed during the 10 min habituation period to the empty arena. The time spent in each chamber was measured via the in-house Machine Vision tracking system and subject's behavior was manually scored.

2.9. Statistical Analyses

Data were plotted using GraphPad Prism 9 Statistical Software (San Diego, CA) and presented as mean ± SEM. Data from the self-grooming and open field assays were analyzed by one-way analysis of variances (ANOVAs) to compare groups. Bonferroni's multiple comparisons post-hoc tests were performed when appropriate. Data from the three-chamber sociability assay were analyzed by multiple unpaired t-tests comparing novel mouse vs. novel object chamber times within groups. Statistical significance was set at $p < 0.05$.

III. Results

3.1. CBD attenuates repetitive behavior in BTBR mice

The repetitive self-grooming assay was used to measure the restricted interest/repetitive behavior phenotype of B6 and BTBR mice (Figure 5). Vehicle-treated BTBR mice spent significantly more time self-grooming (mean = 125 ± 12 s; N = 14) compared to vehicle-treated B6 mice (mean = 49.3 ± 11.3 s; N = 10) ($p = 0.0161$).

Chronic 20 mg/kg CBD dosing in BTBR mice did not alter self-grooming behavior (mean = 128 ± 22 s; N = 12). In the 20 mg/kg CBD-treated BTBR mice, time spent grooming was significantly elevated compared to vehicle-treated B6 mice ($p = 0.0147$) , but not significantly different from vehicle-treated BTBR mice ($p > 0.05$). However, treatment with 50 mg/kg CBD in BTBR mice significantly attenuated the repetitive self-grooming behavior (mean = 57.5 ± 15.7 s; N = 15) compared to vehicle-treated BTB mice ($p = 0.0084$) and 20 mg/kg CBD-treated BTBR mice ($p = 0.0158$). Notably, the reduced levels of grooming time seen in these 50 mg/kg CBD treated mice were comparable to that of the vehicle-treated B6 mice ($p > 0.05$).

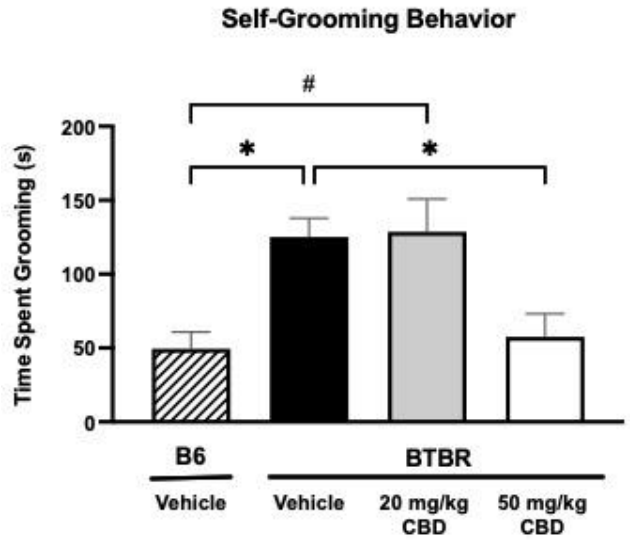

Figure 5. Repetitive behaviors in BTBR mice are attenuated by high dose CBD in self-grooming assay

Vehicle-treated BTBR mice exhibited increased grooming time compared to vehicle-treated B6 controls ($p = 0.0161$). Low dose CBD (ie. 20 mg/kg) treatment had no significant effect on repetitive grooming behavior in BTBR mice, as self-grooming time was similar to vehicle-treated BTBR mice ($p > 0.05$) and significantly higher than vehicle-treated B6 mice ($p = 0.0147$). BTBR mice dosed with 50 mg/kg CBD showed significantly reduced grooming times compared to the vehicle-treated BTBR mice ($p = 0.0168$). Grooming time did not significantly differ between the 50 mg/kg CBD-treated BTBR mice and vehicle-treated B6 mice ($p > 0.05$). Data are presented as mean ± SEM. N = 10-15 mice/group. *$p < 0.05$ compared to vehicle-treated BTBR mice. #$p < 0.05$ compared to vehicle-treated B6 mice.

3.2. CBD rescues social deficits in BTBR mice

The three-chamber assay was used to assess the sociability of BTBR and B6 mice (Figure 6). Vehicle-treated B6 control mice spent more time in the novel mouse chamber compared to the novel object chamber (novel mouse mean = 386 ± 17 s; novel object mean = 162 ± 15 s; SE of difference = 23 s; N = 12) ($p < 0.000001$). In contrast, vehicle-treated BTBR mice displayed diminished sociability, showing no significant preference between the novel mouse and novel object (novel mouse mean = 312 ± 28 s; novel object mean = 238 ± 30 s; SE of difference = 41 s; N = 14) ($p > 0.05$). The 20 mg/kg CBD treatment rescued the social deficits observed BTBR mice as time spent in the novel mouse chamber was significantly greater than time in the novel object chamber (novel mouse mean = 326 ± 22 s; novel object mean = 186 ± 27 s; SE of difference = 35 s; N = 13) ($p = 0.000576$). This rescuing effect was specific to the 20 mg/kg dosage. No significant changes in the asocial behavior of BTBR mice were observed following 50 mg/kg CBD treatment (novel mouse mean = 289 ± 32 s; novel object mean = 227 ± 34 s; SE of difference = 46 s; N = 15) ($p > 0.05$).

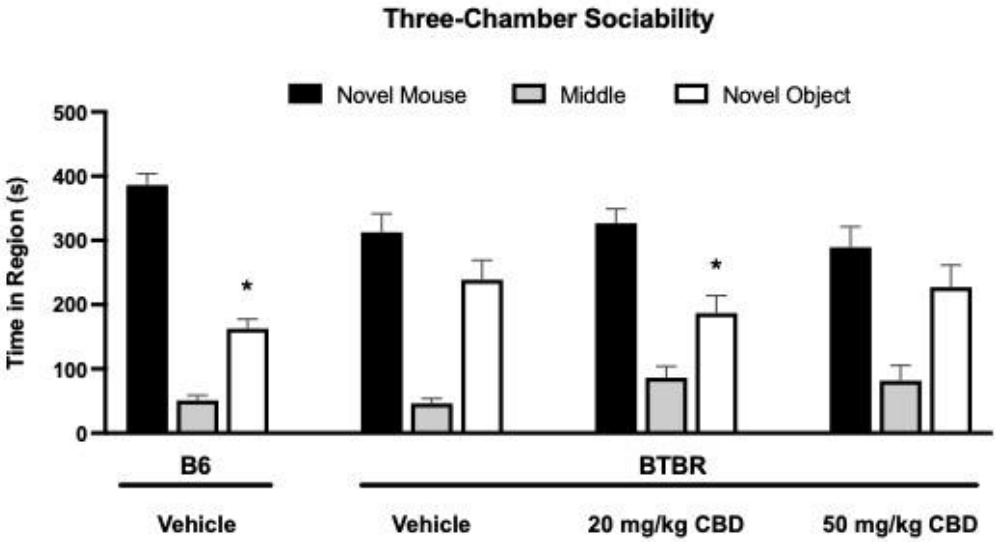

Figure 6. Sociability deficits in BTBR mice are rescued by low dose CBD in 3-chamber sociability assay.

Vehicle-treated B6 mice spent more time in the chamber containing the novel mouse than the chamber containing the novel object ($p < 0.000001$). BTBR mice treated with vehicle demonstrated no social preference for the novel mouse over the novel object ($p > 0.05$), indicating sociability deficits. A social preference for the chamber with the novel mouse vs. the novel object was observed in BTBR mice following 20 mg/kg CBD treatment ($p = 0.000576$). BTBR mice dosed with 50 mg/kg CBD displayed asocial behavior with no difference in time spent in the novel mouse vs. the novel object chambers ($p > 0.05$). Data are presented as mean ± SEM. N = 12-15 mice/group. *$p < 0.05$ within group comparison of novel mouse vs. novel object chamber time.

3.3. CBD reduces hyperlocomotion, but has no effect on anxiety-like behavior in BTBR mice

Because hyperactivity and anxiety are two common ASD co-morbidities [22], exploratory locomotor activity (Figure 7) and anxiety-like behavior (Figure 8) was tested in BTBR and B6 mice using the open field assay. As shown in Figure 3, the distance traveled by vehicle-treated BTBR mice (mean = 6590 ± 220 cm; N = 15) was significantly higher than that of the vehicle-treated B6 mice (mean = 4430 ± 420 cm; N = 15) ($p < 0.0001$). The 20 mg/kg CBD treatment did not appear to have a significant effect on the hyperlocomotor activity, as the distance travelled (mean = 5740 ± 210 cm; N = 11) was similar to that of vehicle-treated BTBR mice ($p > 0.05$) and still significantly higher than that of vehicle-treated B6 mice ($p = 0.0023$). However, the distance travelled by BTBR mice was significantly reduced following the 50 mg/kg CBD treatment (mean = 5170 ± 250 cm; N = 15) compared to vehicle-treated BTBR mice ($p = 0.0003$). Locomotor activity levels were similar in the 50 mg/kg CBD-treated BTBR mice and the vehicle-treated B6 mice ($p > 0.05$).

Anxiety-like behavior was assessed by measuring the duration of time spent near the center of the open field (Figure 8A) vs. the edge (Figure 8B) over the 10 min testing period. Greater time near the perimeter indicated higher levels of anxiety-like behavior [171]. There were no significant differences in time spent in the center of the field or in the edge of the field when comparing B6 controls (center mean = 94.4 ± 11.7 s; edge mean = 505 ± 11 s; N=15) and vehicle-treated BTBR mice (center mean = 87.7 ± 6.7 s; edge mean = 512 ± 6 s; N=15) ($p > 0.05$). Furthermore, CBD treatment had no effect on anxiety-like behavior at either the 20 mg/kg dosage (center mean = 66.8 ± 8.7 s; edge

mean = 533 ± 8 s; N=11) ($p > 0.05$ compared to BTBR vehicle and B6 vehicle) or the 50 mg/kg dosage (center mean = 67.9 ± 6.2 s; edge mean = 532 ± 6 s; N=15) ($p > 0.05$ compared to BTBR vehicle and B6 vehicle).

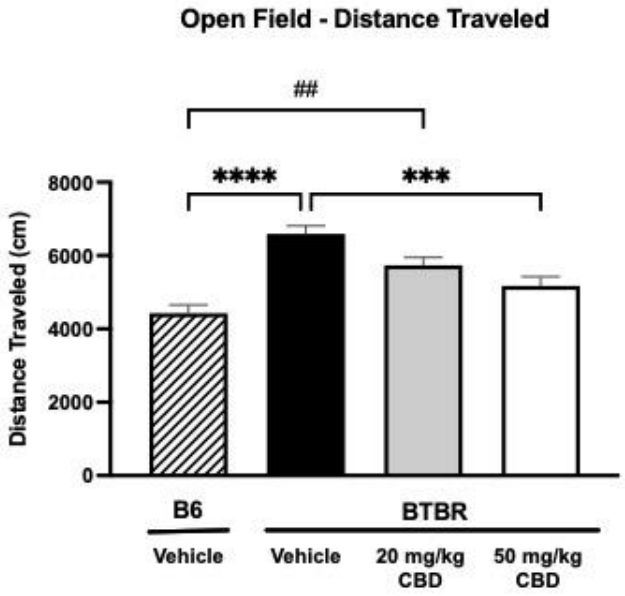

Figure 7. Hyperlocomotion in BTBR mice is reduced by CBD in open field assay

Distance traveled by vehicle-treated BTBR mice was significantly higher than that of B6 controls ($p < 0.0001$), suggesting hyperactivity. BTBR mice dosed with 20 mg/kg CBD travelled distances that were similar to vehicle-treated BTBR mice ($p > 0.05$) and significantly greater than vehicle-treated B6 mice ($p = 0.0023$). 50 mg/kg CBD treatment in BTBR mice significantly reduced locomotor activity compared to vehicle-treated BTBR mice ($p = 0.0003$). There was no significant difference in distance travelled by 50 mg/kg CBD-treated BTBR mice and vehicle-treated B6 mice ($p > 0.05$). Data are presented as mean ± SEM. N = 11-15 mice/group. ***$p < 0.001$ and ****$p < 0.0001$ compared to vehicle-treated BTBR mice. ##$p < 0.01$ compared to vehicle-treated B6 mice.

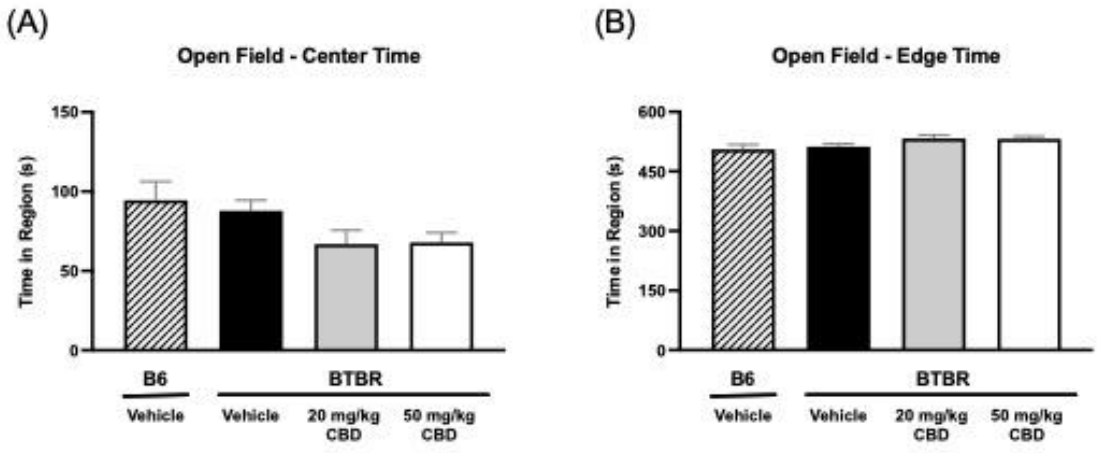

Figure 8. CBD has no effect on anxiety-like behavior in BTBR mice in open field assay

(A) When comparing time spent in the center of the open field, no significant difference was observed between vehicle-treated BTBR and vehicle-treated B6 mice ($p > 0.05$), indicating comparable levels of anxiety. Furthermore, CBD treatment in BTBR mice did not have an effect on center region time for either the 20 mg/kg dose ($p > 0.05$ compared to BTBR vehicle and B6 vehicle) or the 50 mg/kg dose ($p > 0.05$ compared to BTBR vehicle and B6 vehicle). (B) Congruently, time spent in the edge of the open field was similar across all four groups ($p > 0.05$). Data are presented as mean ± SEM. N = 11-15 mice/group.

IV. Discussion

Autism is such a uniquely human condition that developing a well-validated animal model for translational research presents many challenges. Monogenic models of ASD, such as Fragile X Syndrome (FXS) *Fmr1* mutant mice, Tuberous Sclerosis Complex (TSC) *TSC1* or *TSC2* mutant mice and Rett Syndrome *Mecp2* mutant mice, provide good construct validity for exploring potential therapeutic avenues. However, autistic individuals with these single-gene disorders compose only a small subset of the overall autism population, roughly 5-10% [11, 152, 153]. The vast majority of cases are idiopathic in nature [11, 152, 153].

In this study, we investigated the effects of CBD on ASD-associated behavioral phenotypes as recapitulated in the BTBR mouse model of idiopathic autism. This particular animal model of ASD has strong behavioral face validity for the aberrant behaviors examined here, which are thought to be regulated by polygenic mutations also seen in clinical idiopathic ASD cases [68]. Furthermore, this inbred mouse strain has numerous genomic, proteomic, neurophysiologic, anatomic, and synaptic protein elterations which are similar to observations in both monogenic ASD mouse models and ASD patients [68].

The time of initiation and duration of treatment for ASD is a critical factor in long-term patient outcome [18]. Due to evidence of increased benefit with earlier intervention, we chose to begin chronic CBD treatment around the time of weaning on pnd 21 (± 3 days). Although fraught with difficulties, developmental comparisons between species have led researchers to correlate this mouse age to roughly 1-2 human years of age [173]. Following two-week daily i.p. treatment with either vehicle, 20 mg/kg

or 50 mg/kg CBD doses, we investigated the effect of CBD using a battery of behavioral assays that measured repetitive self-grooming and sociability, as well as autism-associated hyperactivity and anxiety.

Excessive barbering and self-grooming, a common characteristic of BTBR mice, is considered similar to the repetitive behaviors seen in autistic individuals. In line with prior studies [161, 174-176], vehicle-treated BTBR mice spent significantly more time self-grooming compared to vehicle-treated B6 mice. We further discovered that this increased repetitive behavior was significantly reduced in BTBR mice receiving high dose (50 mg/kg), but not the low dose (20 mg/kg) CBD treatment. A recent meta-analysis found that current pharmacological treatments for restricted, repetitive behaviors (RRBs) in ASD provide only mild benefits at best [177]. Of the various classes of drugs analyzed, antipsychotics, specifically risperidone and aripiprazole, demonstrated the greatest positive effects [177]. Clinicians, however, need to weigh the minimal benefits against the significant adverse effects associated with antipsychotics, such as metabolic weight gain and fatigue [178]. The data from the current study suggests that CBD, at a proper dose, could be an alternative potential therapeutic agent for the restricted, repetitive behaviors seen in ASD patients.

Risperidone and aripiprazole are currently the only two FDA-approved drugs for the treatment of autism-associated irritability and aggression. These atypical (second generation) antipsychotics have been shown to also improve stereotypy and repetitive behaviors [179-183]. The mechanism of action of risperidone is thought to involve antagonism of the dopamine type 2 (D2) receptors and serotonin type 2A (5-HT_{2A}) receptors; whereas aripiprazole is posited to act via partial agonism/antagonism of D2

and 5-HT_{1A} receptors, along with 5-HT_{2A} receptor antagonism [184, 185]. What could be the mechanism(s) of action of CBD leading to its effects on repetitive behavior? Seeman (2016) demonstrated that CBD displayed partial agonist activity *in vitro* at dopamine D2 receptors in rat striatal tissues [186]. We more recently found CBD to act *in vivo* as a partial agonist on dopamine D2-like receptors in the nematode *Caenorhabditis elegans (C. elegans)* [187]. It is possible that by acting as a partial agonist on dopamine D2 receptor, CBD exhibits its inhibitory effects on repetitive behavior in BTBR mice.

The three-chamber sociability test is commonly used to measure the presence or absence of social preference by comparing the time spent in the chamber with an age-matched, sex-matched novel mouse to the time spent in the chamber with an object (an empty cup). Numerous studies have demonstrated that BTBR mice exhibit reduced social interaction [67, 158, 159]. In this study, we were able to confirm a lower level of sociability in BTBR mice, as compared to their B6 counterparts. In addition, the 20 mg/kg CBD treatment attenuated the social deficit, while no significant effect was observed with the 50 mg/kg CBD treatment. These data suggest that an appropriate dose of CBD may have therapeutic potential to treat the social deficits of ASD patients. This dose-dependent effect on social behavior is supported by a very recent report that an acute single dose of 10 mg/kg CBD, but not 0.1 or 1 mg/kg CBD, was able to enhance social interaction preference in adult BTBR mice [146].

The mesocorticolimbic (MCL) dopaminergic pathway, which consists of the ventral tegmental area, shell and core parts of the nucleus accumbens, and medial prefrontal cortex, is known to mediate social behavior in humans and mice [188]. Previously, it has been reported that selective PPARγ agonists inhibit mesocorticolimbic

dopamine activity and block neuropsychiatric symptoms [189]. Since CBD is a known PPARγ agonist, it is possible that CBD may produce its sociability rescuing effects in BTBR mice by affecting the MCL dopaminergic pathway [189].

ADHD and anxiety disorder are two common ASD co-morbidities [22]. The open field assay is frequently used to test exploratory locomotor activity and anxiety-like behavior in rodents. In the current study, vehicle-treated BTBR mice exhibited hyperlocomotor activity compared to B6 control mice. This finding is in line with numerous published studies [164, 165, 190, 191]. Notably, we further observed a significant, dose-dependent reduction in hyperlocomotor activity in BTBR mice following chronic dosing of CBD. Prior research on anxiety-like behavior in BTBR mice using the open field test have yielded conflicting results, with some demonstrating increased anxiety-like behavior compared to B6 mice [166, 192] and others showing no difference [190, 191]. The age of the BTBR mice tested could in part play a factor in this discrepancy [193]. In this study, time spent in the center of the field was similar between the vehicle-treated BTBR and B6 groups, suggesting comparable levels of anxiety in these strains. Moreover, neither dose of CBD had a significant effect on anxiety-like behavior in the BTBR mice. Together, these open field data suggest that CBD may be a potential treatment for the hyperactivity associated with ASD, but perhaps not effective in attenuating co-occurring anxiety.

In 2018, CBD (Epidiolex) became the first FDA-approved cannabis-derived drug. It is currently indicated for the treatment of seizures associated with Lennox-Gastaut syndrome, Dravet syndrome, and TSC [135]. Reports vary in terms of the comorbid prevalence of epilepsy in patients with ASD, but some studies have estimated close to

50% [194, 195]. Notably, some patients with Dravet Syndrome or TSC display autistic behaviors, and a portion of these individuals are co-diagnosed with ASD [139, 196-198].

The data presented in this study on the effect of CBD in an idiopathic ASD model are consistent with the previous preclinical findings related to Dravet syndrome. Kaplan et al. (2017) demonstrated in the *Scn1a* $^{+/-}$ mouse model of Dravet syndrome that the autism-like social impairments seen in the three-chamber sociability test were improved following acute i.p. injection of CBD at doses of 10 mg/kg and 20 mg/kg [142]. CBD was also able to attenuate seizures at high doses (100 mg/kg and 200 mg/kg) [142]. More recently, Patra et al. (2020) found that twice daily subcutaneous 100 mg/kg CBD injections administered chronically for 4 weeks improved sociability and anxiety-like behavior in this same *Scn1a*$^{+/-}$ mouse model [145]. Therefore, both our data and previous data from studies using animal models of Dravet syndrome demonstrate the potential of CBD for treating ASD symptoms.

In the current study, CBD impacted repetitive behaviors, social deficits, and hyperactivity in the BTBR mice. Human clinical trials testing CBD for the treatment of ASD are currently ongoing (clinicaltrials.gov). Data from pilot studies and case reports, though, are consistent with our preclinical findings and suggest that the cannabinoid may be effective in alleviating both core and comorbid autistic symptoms [147, 150, 151, 157, 199]. The application of a tractable polygenic mouse model of idiopathic ASD such as BTBR mice is significant to answer many questions that have arisen in human clinical trials of CBD. In the future, to help design better clinical trials, more preclinical studies are warranted to determine the dependence of CBD efficacy on dose, sex of the animals, and age of the initiation of treatment.

V. Conclusions

Our understanding of ASD has been ever evolving since Leo Kanner's initial observations of 'infantile autism' in 1943 [16, 200]. Once considered a rare disorder with narrow diagnostic criteria, autism is now acknowledged to be a uniquely heterogenous condition characterized by broad genetic variability and a spectrum of symptomology. The core behavioral hallmarks – social communication deficits and repetitive behavior with restricted interests – can range from mild to severe, and be accompanied by a myriad of comorbidities such as ADHD and anxiety. There is a significant unmet need for the development of therapeutic interventions for ASD which may target the core autistic symptoms. This study demonstrates, for the first time, that repeated CBD dosing in juvenile BTBR mice during the first two weeks post-weaning (ie. pnd21-34) can attenuate autism-related core and comorbid behaviors. Furthermore, our novel findings indicate that these effects by chronic CBD treatment are dose-dependent for different ASD-associated behaviors: for example, 20 mg/kg CBD was effective for rescuing sociability deficits, whereas 50 mg/kg CBD was effective for reducing repetitive behaviors and hyperlocomotor activity. Together, this study indicates the therapeutic efficacy of CBD in a preclinical model of idiopathic ASD, and suggests that the proper dosage of CBD administered chronically beginning at an early age may be clinically useful in targeting both core and comorbid symptoms in ASD patients.

CHAPTER III

ALTERATIONS IN IMMUNE CELL POPULATIONS IN A MODEL OF IDIOPATHIC AUTISM SPECTRUM DISORDER

I. Introduction

Autism Spectrum Disorder (ASD) is a heterogenous group of neurodevelopmental disabilities characterized by impaired social interactions, communication deficits, and restricted interests and repetitive behavior [1]. The prevalence of the disorder has drastically risen over the last two decades, currently affecting 1 in 36 children, according to the CDC [4]. At present, standard of care relies heavily on behavioral therapy with varying success among this heterogenous patient population [201]. There is a significant unmet need for a pharmacological intervention for the treatment of core autistic symptoms. With ASD gradually becoming one of the most common neurodevelopmental disorders, ongoing research is crucial to better understanding the complex factors at play in ASD and to developing more effective therapies for individuals affected by the disorder.

Our knowledge of the molecular mechanisms underlying ASD pathogenesis remains limited. The vast majority of cases are idiopathic, with rare genetic variants, including *de novo* mutations, copy number variants and monogenic disorders such as Fragile X Syndrome and Rett Syndrome, accounting for ~20-30% of ASD patients [11, 152, 153]. Based on twin and familial epidemiological studies, the etiology of ASD is understood as a complex interaction between diverse genetic, epigenetic and

environmental risk factors [7-10]. Although this multifaceted etiology poses clear pharmacological challenges, strides have been made toward identifying specific therapeutic targets with the use of functional genomics. GWAS studies have revealed common themes among ASD-related gene clusters with convergence of signaling pathways involved in immune system regulation and synaptic function [13].

There is clinical evidence for dysregulation in both the innate and adaptive branches of the immune system in ASD patients [202, 203]. Researchers have found increased activation of circulating peripheral blood mononuclear cells (PBMCs) and a marked pro-inflammatory cytokine profile, both of which are associated with greater behavioral impairments in children with ASD [27, 204, 205]. Furthermore, immunohistochemical and ELISA analysis of post-mortem brains and cerebral spinal fluid (CSF) of ASD patients showed evidence of chronic neuroinflammation, increased microglial activation, and a distinct pro-inflammatory cytokine/chemokine profile as well [206-209].

The use of immune cell and cytokine levels as diagnostic and treatment biomarkers for ASD has been suggested by multiple researchers [27-30]. Developing a more comprehensive profile of immune alterations associated with ASD may help in better understanding the neurodevelopmental and behavioral changes observed in patient subpopulations and animal models, as well as identifying pharmacological targets for new drug therapies.

In this study, we sought to characterize the immune cells in the peripheral tissues and brains of BTBR $T^{+}Itpr3^{tf}/J$ (BTBR) mice using flow cytometry. BTBR mice have become an established model of idiopathic ASD, in part due to their core behavioral

deficits, unusual pattern of ultrasonic vocalizations, neuroanatomical abnormalities, and aberrant immune response [68]. This aberrant immune phenotype is similar to that seen clinically in patients with ASD, including alterations in immune cell populations, inflammatory response, and neuroimmunomodulation [72]. As such, the BTBR model is ideally suited for our investigation into immunological alterations associated with ASD.

In the periphery, we focused on both αβ and γδ T cell subpopulations in four different tissues: blood, spleen, mesenteric lymph nodes (MLN) and cervical lymph nodes (CLN). The former subset, composed of $CD4^+$ and $CD8^+$ T cells, is the most abundant and well-studied of the $CD3^+$ T lymphocytes [210]. Given their importance in modulating adaptive immune responses and balancing inflammatory-anti-inflammatory signaling, it is unsurprising that dysregulation in $CD4^+$ and $CD8^+$ T cells can have wide spread implications [211]. While less research has been devoted to γδ T cells, emerging evidence has revealed their importance in broad antigen recognition, autoimmune response regulation and tissue homeostasis [212, 213]. Alterations in αβ and γδ T cell expression, activation states, and associated cytokine production have been previously implicated in various clinical neuropathologies, including ASD [214-219]. While there are some reports of changes in the αβ T cell profiles of BTBR mice [73, 220, 221], previous investigations have not conducted in the four different tissues simultaneously. Most importantly, to our knowledge, γδ T cells have not been investigated in this murine model of idiopathic ASD.

We also assessed in these peripheral T cell populations expression of two different cytokines: IL-17a and IFN-γ. Both are known to perform crucial functions in protective immunity, but can also contribute to immunopathologies when signaling is

disrupted [222-225]. Alterations in the expression of inflammatory cytokines, including IL-17a and IFN-γ, have been reported in some subsets of ASD cases [226-228], but their precise roles in ASD pathogenesis are still under investigation. Preclinical studies in B6 mice have pointed to the importance of meningeal IL-17a [229] and IFN-γ [230] signaling in regulating social behavior and anxiety-like behavior, respectively. It remains unknown whether IL-17a or IFN-γ dysregulation could be a potential contributor to the sociability deficits or comorbid anxiety observed in both autistic patients and BTBR mice.

In whole brain samples, we examined microglial cells, the resident immune cells in the central nervous system (CNS), which play a crucial role in immune surveillance and brain homeostasis [231]. We also measured expression of the microglial marker, triggering receptor expressed on myeloid cells 2 (TREM2), as it is important for synaptic pruning, neurotransmission and long-range functional connectivity, as well as sociability [232]. Notably, in post-mortem brain samples from individuals with ASD, a negative correlation was observed between TREM2 levels and the severity of ASD symptoms [232]. To our knowledge, TREM2 expression in BTBR mice has not yet been investigated.

Overall, the two main goals of this study were: first, to characterize the T cell subpopulations and cytokine profiles in the blood and peripheral lymphoid organs, and second, to characterize TREM2 expression in microglia – both using the BTBR mouse model of idiopathic ASD.

II. Materials and Methods

2.1. Materials and Reagents

Isoflurane (VetOne Fluriso) was purchased through the University of Louisville Comparative Medicine Research Unit. Fluorescence-activated cell sorting (FACS) Buffer containing 1X Phosphate Buffered Saline (PBS) pH 7.4 and 2% Bovine Serum Albumin (BSA) (Sigma Aldrich, St Louis, MO) was prepared fresh for each experiment. A glass tissue grinder pestle was purchased from Corning (Manassas VA) and 70 μm cell strainers were purchased from VWR (Radnor, PA). RBC Lysis buffer was purchased from BioLegend (San Diego, CA) and 500 mM EDTA was purchased from Sigma Aldrich. 23-guage BD PrecisionGlide needles were purchased from Fischer Scientific (Waltham, MA) and Percoll was purchased from Sigma Aldrich. RPMI 1640 Medium and 5mL round bottom polystyrene FACS test tubes were purchased from Corning. For stimulation of single cell suspensions, brefeldin A (BFA), Phorbol 12-myristate 13-acetate (PMA), and Ionomycin were all purchased from Sigma Aldrich. All monoclonal Abs used are listed in Table 1. For Fc receptor blocking, anti-mouse CD16/32 (93) was purchased from BioLegend. The following fluorescent tag-conjugated monoclonal Abs were used for cell-surface staining: anti-CD3 PerCP-eFluor710 (500A2) (Invitrogen, Waltham, MA), anti-CD4 FITC (GK1.5) (BioLegend), anti-CD4 eFluor506 (RM4-5) (Invitrogen), anti-CD8 Brilliant Violet 605 (53-6.7) (BioLegend), anti-γδ TCR PE (GL3) (BioLegend), anti-CD45 BUV737 (30-F11) (BD Biosciences, San Jose, CA), anti-CD11b PE/Cy7 (M1/70) (BioLegend), and TREM2 FITC (78.18) (Invitrogen). The following fluorescent tag-conjugated monoclonal Abs were used for intracellular staining: anti-IL-17a Brilliant Violet 421 (TC11-18H10.1) (BioLegend) and anti-INF-γ Alexa Fluor 647

(XMG1.2) (BioLegend). The Invitrogen (Carlsbad, CA) eBioscience Intracellular Fixation and Permeabilization Buffer Set was used for sample fixation prior to intracellular Ab staining. UltraComp eBeads™ Compensation Beads and CountBright™ Plus Absolute Counting Beads were both purchased from Invitrogen.

2.2. Animal Maintenance and Housing

This study was conducted at University of Louisville School of Medicine. All experiments were performed according to a protocol approved by the University of Louisville Institutional Animal Care and followed committee and NIH guidelines. Subjects were offspring of C57BL/6J (B6) and BTBR T+Itpr3tf/J (BTBR) breeding pairs obtained from Jackson Laboratory (Bar Harbor, ME). Animals were bred and housed in clean, federally regulated and AAALAC-accredited facilities operated by the University of Louisville School of Medicine Department of Animal Care. Mice were weaned at post-natal day 21 and no more than five littermates were housed per cage (33 cm long x 19 cm wide x 15 cm high) with free access to food and water, in a humidity (30%) and temperature (22 ± 1°C) controlled room with a 12 h light/dark cycle (lights on at 06:00 A.M.). All animals utilized in this project were monitored daily for evidence of discomfort, distress, pain or injury.

2.3. Tissue Collection & Single Cell Suspension

Male BTBR and B6 mice between the ages of 8 and 12 weeks old were anesthetized with isoflurane prior to collection of blood, peripheral lymphoid organs and whole brains.

Whole blood samples were obtained via cardiac puncture. Approximately 500 μL of blood was drawn from the heart using a 23-guage needle and transferred to tubes containing 50 μL 500 mM EDTA, to prevent clotting. 5 mL RBC lysis buffer was added to each tube, and allowed to incubate at RT x 10 min. 10 mL FACS buffer was added to each tube to stop the reaction and tubes were centrifuged at 4°C x 4 min at 500g. The pellet was resuspended in 5mL RBC lysis buffer and again allowed to incubate at RT x 10 min, before 10 mL FACS buffer was added to each tube and tubes were centrifuged 4°C x 4 min at 500g. The supernatant was discarded, cells were resuspended in 5 mL FACS buffer and cells were counted.

Whole spleens were homogenized in FACS buffer using a glass tissue grinder pestle, filtered through a 70 μm cell strainer, and centrifuged at 4°C x 7 min at 500g. The supernatant was discarded and then spleen cells were resuspended in 3 mL RBC lysis buffer and incubated x 4 min. 10 mL FACS buffer was added to each tube to stop the reaction and tubes were centrifuged at 4°C x 4 min at 500g. The supernatant was discarded, cells were resuspended in 5 mL FACS buffer and cells were counted.

Mesenteric lymph nodes and superficial cervical lymph nodes were homogenized in FACS buffer using a glass tissue grinder pestle, filtered through a 70 μm cell strainer and centrifuged at 4°C x 7 min at 500g. The supernatant was discarded, cells were resuspended in 5 mL FACS buffer and cells were counted.

Whole brains were harvested from anesthetized BTBR and B6 mice following transcardial perfusion using phosphate-buffered saline (50 mL/mouse). Brain tissues were homogenized in FACS buffer using a glass tissue grinder pestle, filtered through a 70 μm cell strainer, and centrifuged at 4°C x 7 min at 500g. Supernatant was discarded, the

pellet was resuspended in 5 mL 25% Percoll solution, and centrifuged at 18°C x 30 min at 521g to remove myelin and debris. The supernatant was discarded and the pellet was resuspended in 10 mL FACS buffer. The suspension was transferred to a new tube and centrifuged at 4°C x 4 min at 500g. The supernatant was discarded, cells were resuspended in 5 mL FACS buffer and cells were counted.

2.4. Stimulation of Single Cell Suspensions

For each sample, $5x10^6$ cells were suspended in 1 mL RPMI 1640 medium and plated on a 24-well plate. In order to evaluate intracellular cytokine expression, 6.9 μL of stimulation mixture (5 μL BFA, 0.5 μL PMA, 1.4 μL Ionomycin) was added to each well and plates were incubate at 37°C x 4-5 h.

2.5. Antibody Staining & Flow Cytometry

Following stimulation, samples were transferred to 5 mL FACS round bottom tubes and centrifuged at 4°C x 4 min at 500g. The supernatant was discarded and cells were resuspended in 50 μL FACS buffer. All cell samples were incubated with anti-mouse CD16/32 (93) (BioLegend) x 10 min to block Fc receptors. Next, a fluorescent tag-conjugated antibody mixture, containing anti-CD3, anti-CD4, anti-CD8, anti-TCRγδ, anti-CD45 and/or anti-CD11b, was added to cells and allowed to incubate x 12 min at RT. Samples were washed with 1 mL FACS buffer, centrifuged 4°C x 4 min at 500g, and the supernatant was discarded. Prior to intracellular Ab staining, cells were fixed using the Invitrogen eBioscience Intracellular Fixation and Permeabilization Buffer Set, according to manufacture instructions. Briefly, cell pellets were resuspended in 200 μL of

fixation mixture (1 part Fix/Perm concentrate: 3 parts eBioscience) and allowed to incubate at 4°C x 17 min. Samples were then washed with 1 mL of Permeabilization buffer, centrifuged at 4°C x 4 min at 500g, and the supernatant was discarded. Cells were resuspended in 50 μL permeabilization buffer. Next, a fluorescent tag-conjugated antibody mixture containing anti-IL-17a and anti-INF-γ was added to cells and samples incubated at 4°C overnight. Samples were washed with 1 mL of Permeabilization buffer, centrifuged at 4°C x 4 min at 500g, and the supernatant was discarded. Cells were resuspended in 400 μL permeabilization buffer. Stained cells were analyzed on a BD LSRFortessa (BD Biosciences). Flow cytometry data were analyzed using FCS Express 7 De Novo software. Representative gating strategies for peripheral tissues and whole brains are shown in Figures 9 and 15, respectively.

2.6. Statistical Analysis

7-12 mice were used per strain per tissue. Using GraphPad Prism 9 Statistical Software (San Diego, CA), data were analyzed by unpaired two-tailed Student's t test. Data are presented as mean ± SEM. A p-value < 0.05 was considered significant. For all figures: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Table 1. Flow cytometry monoclonal antibodies used

Flow Cytometry Antibodies				
Target	**Format**	**Clone**	**Source**	**Catalog No.**
CD16/32		93	BioLegend	101302
CD3e	PerCP-eFluor710	500A2	Invitrogen eBioscience	46-0033-82
CD4	FITC	GK1.5	BioLegend	100406
CD4	eFluor506	RM4-5	Invitrogen eBioscience	69-0042-80
CD8	Brilliant Violet 605	53-6.7	BioLegend	100744
γ/δ TCR	PE	GL3	BioLegend	118108
CD45	BUV737	30-F11	BD Biosciences	748371
CD11b	PE/Cy7	M1/70	BioLegend	101215
TREM2	FITC	78.18	Invitrogen	MA5-28223
IL-17a	Brilliant Violet 421	TC11-18H10.1	BioLegend	506926
INF-γ	Alexa Fluor 647	XMG1.2	BioLegend	505814

III. Results

3.1. CD3$^+$ T Cells

The gating strategy used for flow cytometric analysis of peripheral tissues is shown in Figure 9. In order to identify T lymphocytes, singlets were stained with the CD3 marker (Figure 10). BTBR mice expressed a significantly higher percentage of CD3$^+$ cells in the blood (p=0.0048), spleen (p=0.0101), MLN (p<0.0001), and CLN (p=0.0166) (Figure 6A). For all four tissue types, the number of CD3$^+$ T cells was also higher in BTBR mice compared to B6 controls (blood p=0.0133; spleen p=0.0045; MLN p<0.0001; CLN p=0.0166) (Figure 6B).

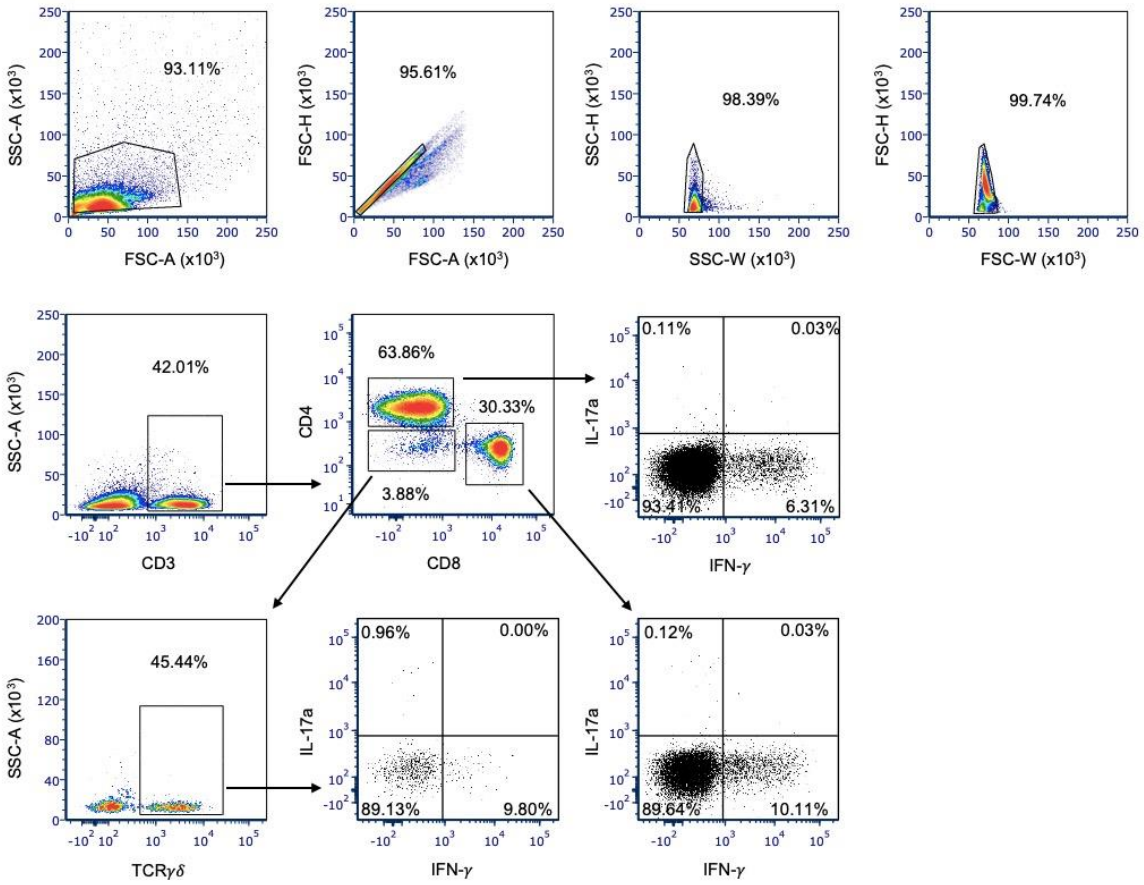

Figure 9. Representative flow cytometric gating strategy for peripheral tissues

For peripheral lymphoid organs and peripheral blood from B6 and BTBR mice, cells were first loosely gated on SSC-A vs FSC-A plot. Next, doublets and additional cellular debris were excluded by FSC-H vs FSC-A gating, followed by SSC-H vs SSC-W gating and FCS-H vs FSC-W gating. T cells were then identified by expression of CD3. To identify subsets of T lymphocytes, the $CD3^+$ T cells were then gated for expression of CD4 and CD8. The $CD3^+$ T cells that were $CD4^-CD8^-$ double negative were gated for expression of TCRγδ. $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD3^+CD4^-CD8^-TCR\gamma\delta^+$ cells were all gated for expression IL-17a and IFN-γ. Example shown is of BTBR spleen sample.

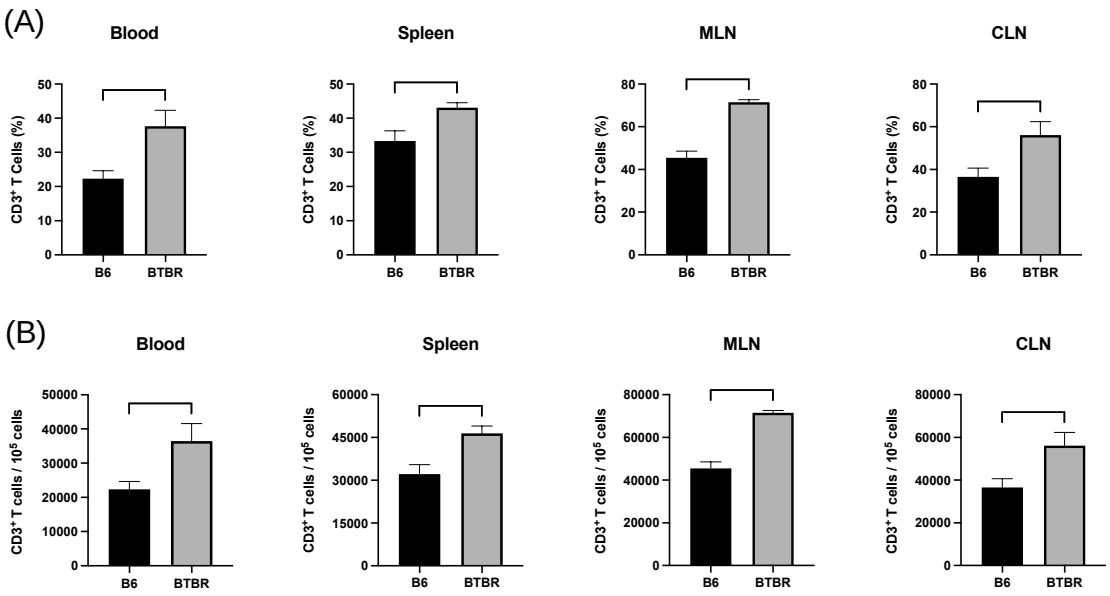

Figure 10. Peripheral CD3$^+$ T cell profile in BTBR and B6 mice

Frequencies (A) and numbers (B) of CD3$^+$ T cells in the blood, spleen, MLN and CLN of B6 and BTBR mice are shown. 7-12 mice were used per strain per tissue. Data are presented as mean ± SEM. *p* values, as determined by unpaired two-tailed Student's t test, were considered significant if <0.05. **p* < 0.05, ***p* < 0.01, and *****p* < 0.0001.

3.2. $CD4^+$ and $CD8^+$ T Cells

We next wanted to examine the subsets that make up this increased population of $CD3^+$ T cells in BTBR mice. Both the percentage (Figure 11A) and number (Figure 11B) of $CD4^+$ T cells were increased in the blood (% $p<0.0001$, # $p=0.0003$), spleen (% $p<0.0001$, # $p=0.0004$), MLN (% $p<0.0001$, # $p<0.0001$), and CLN (% $p<0.0001$, # $p=0.0008$) of BTBR mice, compared to B6 mice. In contrast, the percentage of $CD8^+$ T cells (Figure 12A) was significantly lower in all four tissue types of BTBR mice: blood ($p<0.0001$), spleen ($p<0.0001$), MLN ($p<0.0001$), and CLN ($p<0.0001$). Notably, the number of $CD8^+$ T cells (Figure 12B), however, did not significantly differ between BTBR and B6 controls: blood ($p=0.7061$), spleen ($p=0.4045$), MLN ($p=0.2234$), and CLN ($p=0.7632$).

Given the increase in percentage of $CD4^+$ T cells and decrease in percentage of $CD8^+$ T cells, it thus makes sense that the $CD4^+/CD8^+$ ratio is higher in the blood ($p<0.0001$), spleen ($p<0.0001$), MLN ($p<0.0001$), and CLN ($p<0.0001$) of BTBR mice (Figure 13).

No significant differences in IL-$17a^+$ expression were observed in any of the tissues for either $CD4^+$ T cells ($CD4^+$IL-$17a^+$ blood $p=0.8566$; spleen $p=0.3893$; MLN $p=0.1403$; CLN $p=0.9008$) (Figure 11C) or $CD8^+$ T cells ($CD8^+$IL-$17a^+$ blood $p=0.3456$; spleen $p=0.0597$; MLN $p=0.8337$; CLN $p=0.1333$) (Figure 12C). Interestingly, BTBR have a significantly higher levels of IFN-γ-producing CD4+ T cells in the spleen ($p=0.0113$) (Figure 11D) and significantly lower levels of IFN-γ-producing CD8+ T cells in the MLN ($p=0.0309$) (Figure 12D) when compared to B6 mice. No other differences were observed in IFN-γ-producing CD4+ T cells (blood $p=0.1139$; MLN $p=0.0778$; CLN

p=0.3483) (Figure 11D) or IFN-γ-producing CD8+ T cells (blood *p*=0.2181; spleen *p*=0.4856; CLN *p*=0.3072) (Figure 12D).

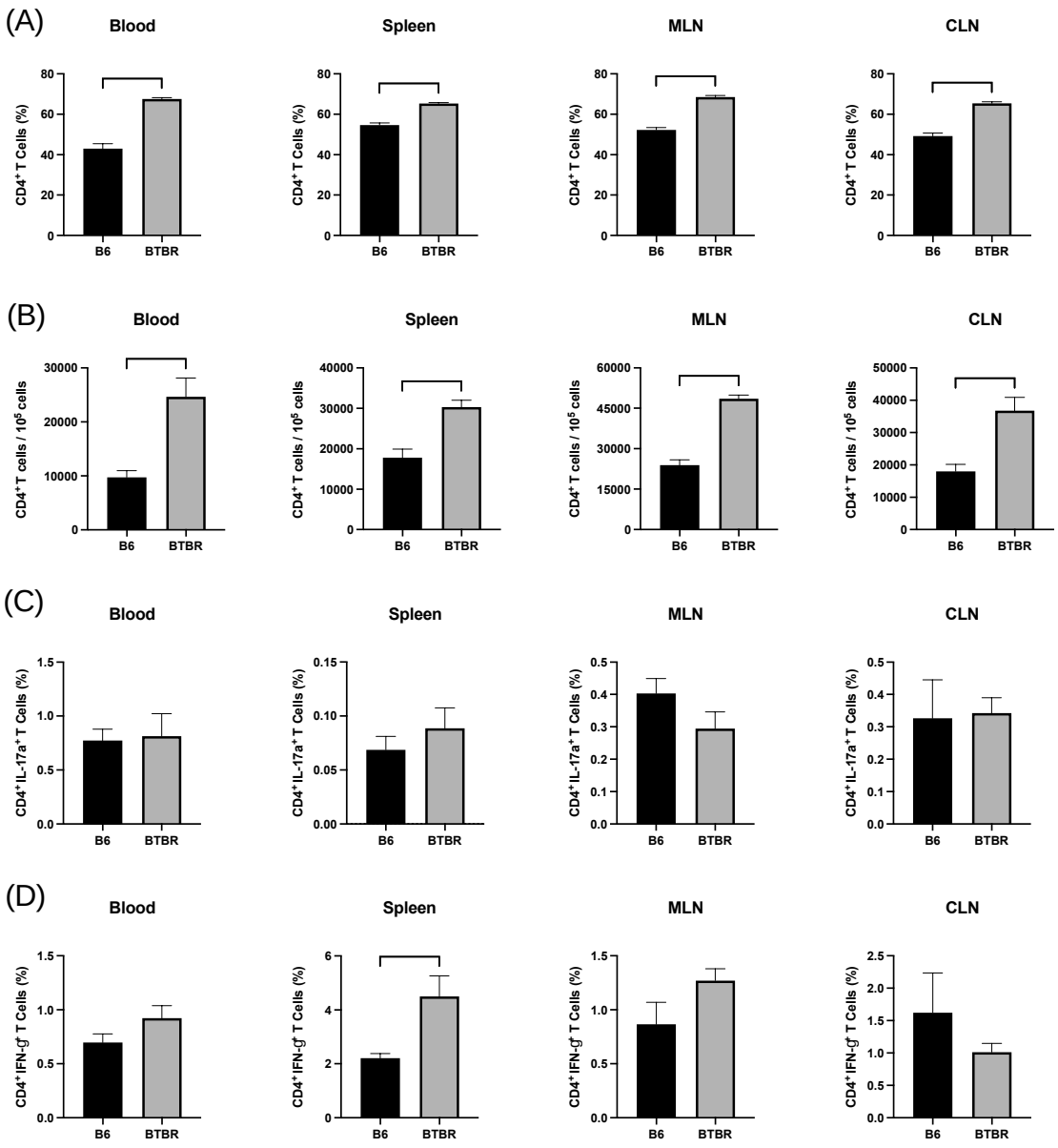

Figure 11. Peripheral CD4$^+$ T cell profile in BTBR and B6 mice

Frequencies (A) and numbers (B) of CD4$^+$ T cells, frequencies of CD4$^+$IL-17a$^+$ T cells (C), and frequencies of CD4$^+$IFN-γ^+ T cells (D) in the blood, spleen, MLN and CLN of B6 and BTBR mice are shown. 8-12 mice were used per strain per tissue. Data are presented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$, and ****$p < 0.0001$ indicate significant difference between the two strains.

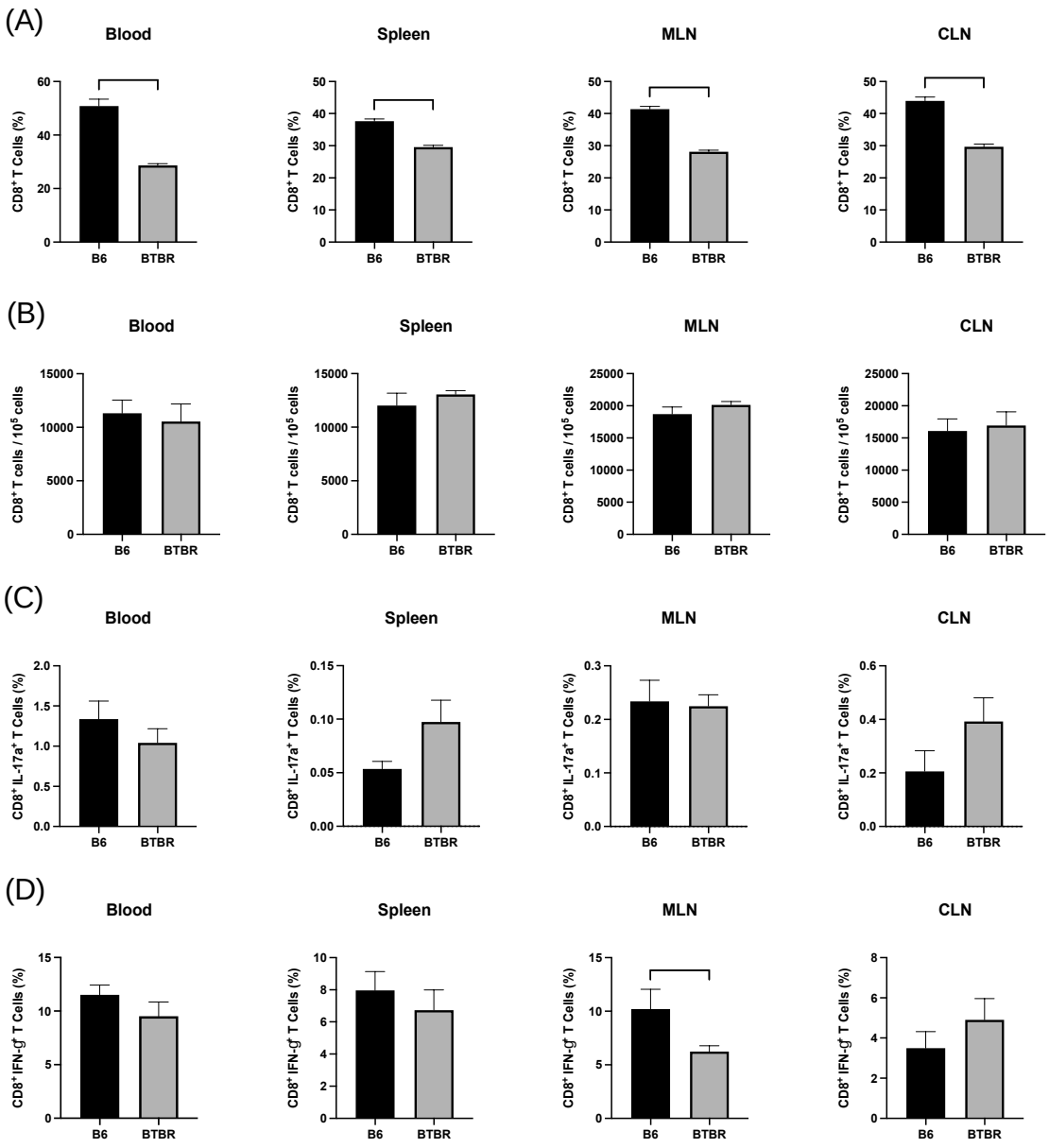

Figure 12. Peripheral CD8$^+$ T cell profile in BTBR and B6 mice

Frequencies (A) and numbers (B) of CD8$^+$ T cells, frequencies of CD8$^+$IL-17a$^+$ T cells (C), and frequencies of CD8$^+$IFN-γ^+ T cells (D) in the blood, spleen, MLN and CLN of B6 and BTBR mice are shown. 8-12 mice were used per strain per tissue. Data are presented as mean ± SEM. *$p < 0.05$ and ****$p < 0.0001$ indicate significant difference between the two strains.

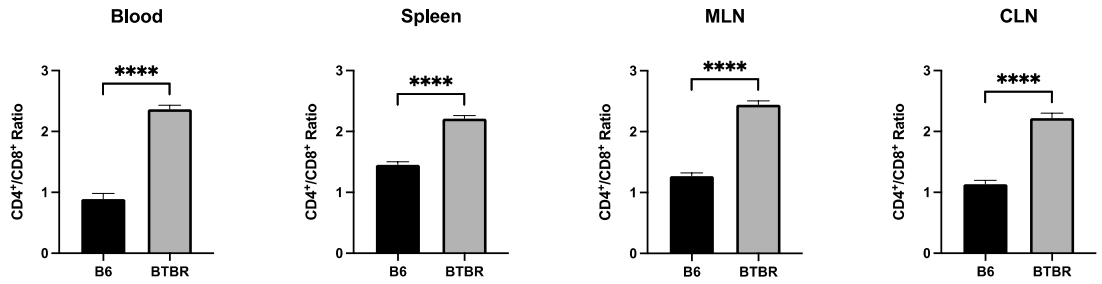

Figure 13. $CD4^+/CD8^+$ T Cell Ratio in BTBR and B6 mice

$CD4^+/CD8^+$ ratio in the blood, spleen, MLN and CLN of B6 and BTBR mice are shown. 8-12 mice were used per strain per tissue. Data are presented as mean ± SEM. ****$p <$ 0.0001 indicates significant difference between the two strains.

3.3. TCRγδ⁺ T Cells

γδ T cells have been studied considerably less than their alpha beta counterparts. We found significantly lower percentages of TCRγδ⁺ T cells in the blood ($p=0.0185$), spleen ($p=0.0072$), and MLN ($p<0.0001$) (Figure 14A). However, in all three, the number of TCRγδ⁺ T cells in BTBR mice did not differ significantly from the number in B6 mice: blood ($p=0.6085$), spleen ($p=0.2982$), and MLN ($p=0.9331$) (Figure 14B). In the CLN, neither the frequency ($p=0.0656$) (Figure 14A) nor number ($p=0.0527$) (Figure 14B) of TCRγδ⁺ T cells differed between BTBR and B6 mice.

In BTBR mice, the percentage of TCRγδ⁺IL-17a⁺ cells was lower in the blood ($p=0.0386$), spleen ($p=0.0005$) and CLN ($p=0.0248$), but not the MLN ($p=0.2279$) (Figure 14C). The percentage of TCRγδ⁺IFN-γ⁺ cells was lower in the blood ($p=0.0023$), spleen ($p=0.0166$), and MLN ($p=0.0188$), but not the CLN ($p=0.4755$) of BTBR mice compared to B6 mice (Figure 14D).

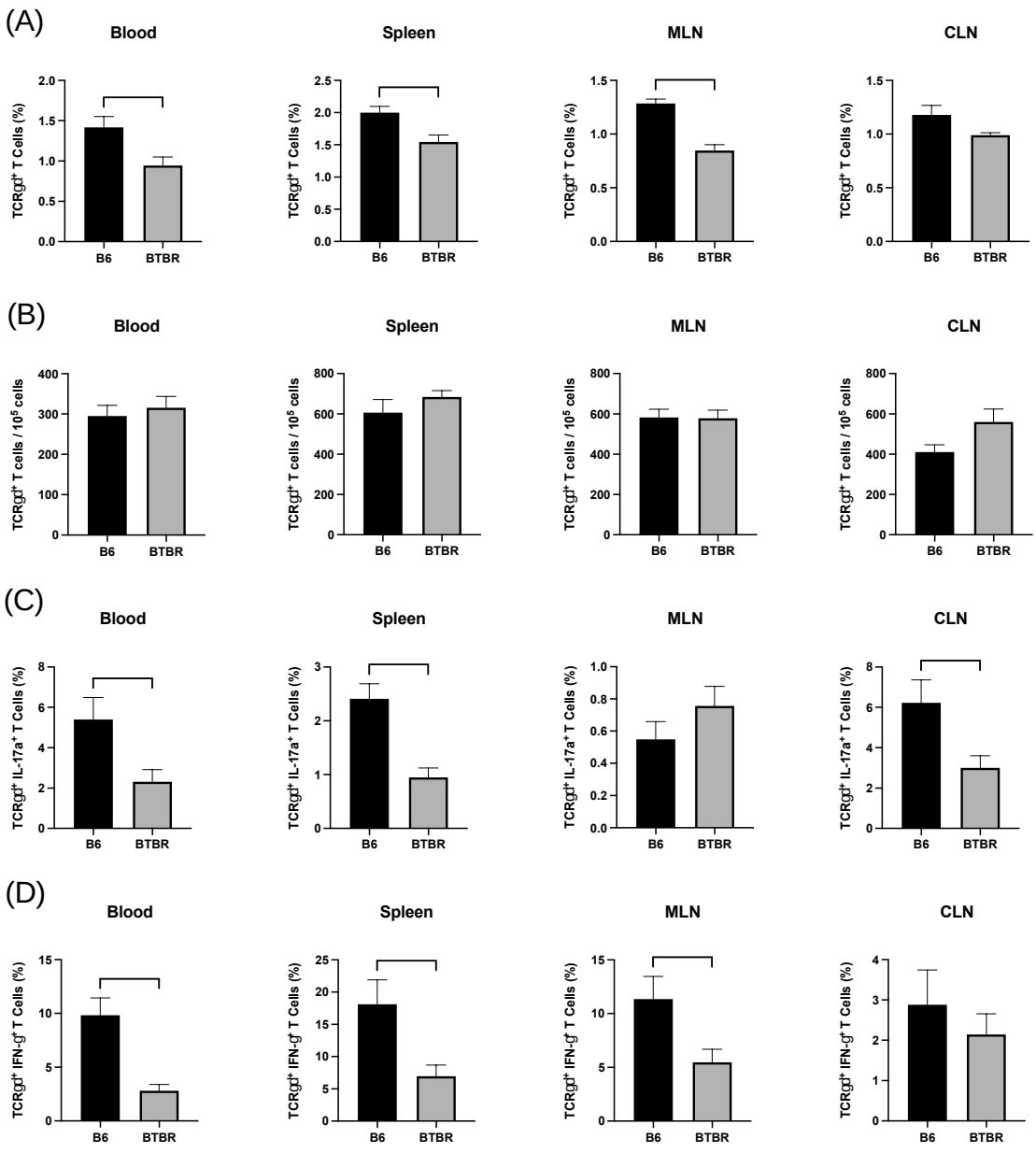

Figure 14. Peripheral TCRγδ$^+$ T cell profile in BTBR and B6 mice

Frequencies (A) and numbers (B) of TCRγδ$^+$ T cells, frequencies of TCRγδ$^+$IL-17a$^+$ T cells (C), and frequencies TCRγδ$^+$IFN-γ$^+$ T cells (D) in the blood, spleen, MLN and CLN of B6 and BTBR mice are shown. 8-12 mice were used per strain per tissue. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ indicate significant difference between the two strains.

3.4. $CD11b^{+}CD45^{low}$ Microglia and $TREM2^{+}$-Expressing Microglia

The gating strategy for whole brain samples shown in Figure 15 outlines how we performed flow cytometric analysis to identify microglia and their expression of TREM2 (Triggering Receptor Expressed on Myeloid Cells 2), a protein primarily expressed on the surface of microglia cells. We found no difference in the percentage (Figure 16A) or number (Figure 16B) of $CD11b^{+}CD45^{low}$ microglia cells in B6 and BTBR brains (% p=0.5550, # p=0.3645). However, both the percentage (Figure 16C) and number (Figure 16D) of $TREM2^{+}$ $CD11b^{+}CD45^{low}$ microglia cells was lower in BTBR mice compared to B6 mice (% p=0.0001, # p=0.0094).

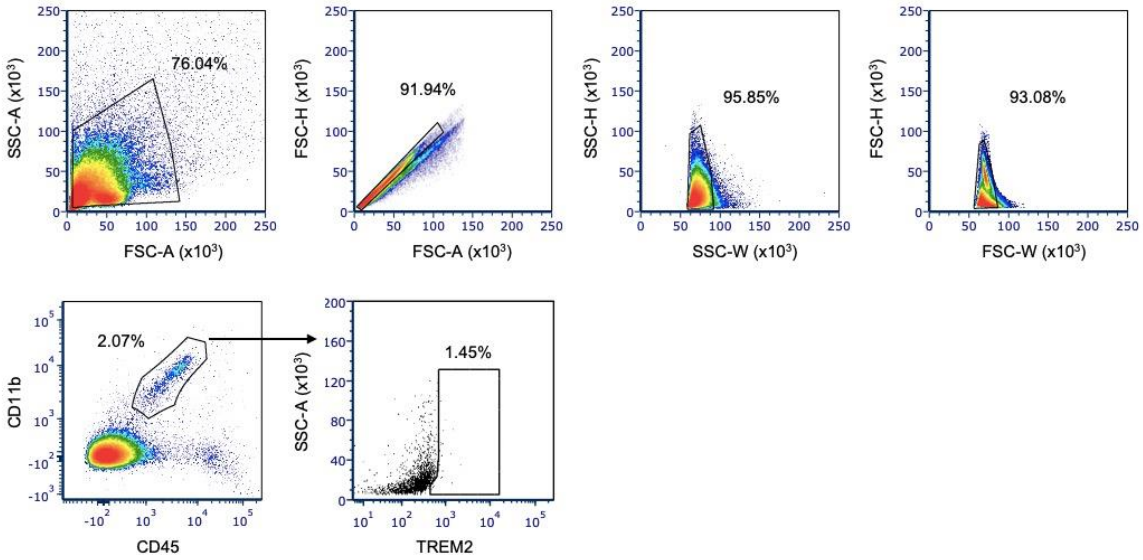

Figure 15. Representative flow cytometric gating strategy for whole brains

For whole brains from transcardially-perfused B6 and BTBR mice, cells were first loosely gated on SSC-A vs FSC-A plot. Next, doublets and additional cellular debris were excluded by FSC-H vs FSC-A gating, followed by SSC-H vs SSC-W gating and FCS-H vs FSC-W gating. A CD11b vs CD45 plot was used to identify $CD11b^{+}CD45^{low}$ microglia cells, which were then assessed for expression of TREM2. Example shown is from BTBR brain sample.

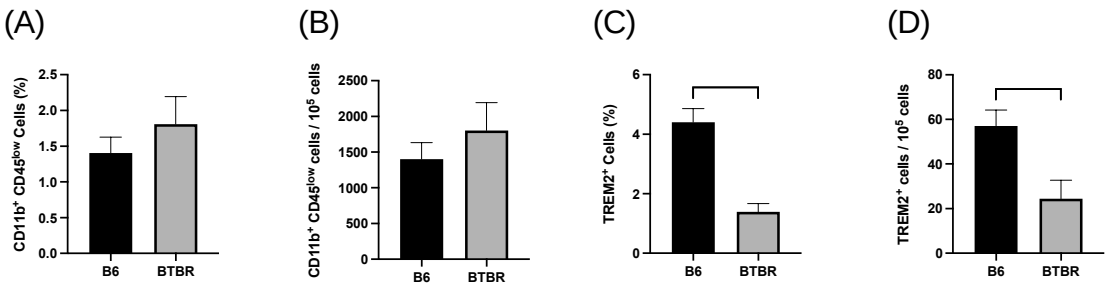

Figure 16. Microglia and TREM2 expression in whole brains of BTBR and B6 mice

Frequencies (A) and numbers (B) of $CD11b^{+}CD45^{low}$ microglial cells, and frequencies (C) and numbers (D) of TREM2-expressing $CD11b^{+}CD45^{low}$ microglial cells in brains of B6 and BTBR mice. 7-9 mice were used per strain. Data are presented as mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ significant difference between the two strains.

IV. Discussion

Autism Spectrum Disorder is a uniquely human condition characterized by social communication deficits and repetitive behaviors with restricted interests. While the underlying etiology of ASD remains largely unknown, emerging evidence suggests a potential involvement of the immune system in its pathogenesis [13, 202, 203]. Responsible for protecting the body against harmful pathogens, the immune system also plays a crucial role in brain development and function [233, 234]. Aberrations in various immune components, such as immune cell populations, cytokines, and chemokines, have been observed in individuals with ASD [27, 202-209, 235, 236]. Much remains to be elucidated on whether these immune alterations may contribute to the disrupted neurodevelopment and atypical brain connectivity associated with clinical autism.

Given the highly heterogenous nature of autism, many challenges arise when developing a well-validated animal model for translational research that recapitulates the complex immunological alterations observed clinically. The BTBR model is ideally suited for our investigation due to its strong immunological face validity. The aberrant immune phenotype observed in BTBR mice closely resemble those seen in ASD patients, such as alterations in immune cell populations, inflammatory response, and neuroimmunomodulation [68, 72]. In this study, we aimed to characterize the immune profile in the BTBR mice model through flow cytometric analysis, so as to better understand the potential role of T cells and microglial cells in immune dysregulation associated with idiopathic ASD.

As an essential component of the adaptive immune system, T lymphocytes play a significant role in maintaining immune homeostasis in both the periphery and CNS

through the recognition of foreign antigens, cell-mediated immunity, helper functions, and immunological (T cell) memory [210, 237]. Cluster of Differentiation 3 (CD3) is a multiprotein complex that functions as a co-receptor for T cells, forming a noncovalent association with the T cell receptor (TCR) and assisting in T cell activation [238]. The CD3 complex is a defining feature in T lymphocyte lineage and its cell-surface expression is a key marker for identifying T cells [238]. Consistent with previous reports [73, 221, 239], we found that levels of $CD3^+$ T cells in BTBR mice are elevated compared to B6 mice. This increased percentage and total number of T lymphocytes were observed in all four peripheral tissues examined – blood, spleen, MLN and CLN. As such, we wanted to gain further insight into which specific T cell subsets were also affected in these mice.

$CD3^+$ T lymphocytes can be broadly categorized into two main groups based on their TCR expression: TCRαβ and TCRγδ [240]. αβ T cells, which make up the majority of all circulating T cells (~95%), are involved in recognizing peptide antigens presented by major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs), such as dendritic cells, macrophages, and B cells [240, 241]. Their primary responsibility involves mediating immune responses against viral, bacterial, and intracellular pathogens, as well as cancer cells [240]. αβ T cells can be further classified into subsets based on expression of co-receptors CD4 and/or CD8 [210]. $CD4^+$ T cells, also known as helper T cells, play a central role in orchestrating immune responses by regulating the activities of other immune cells, such as B cells, cytotoxic T cells, and macrophages [210]. In contrast, $CD8^+$ T cells, commonly referred to as cytotoxic T cells, directly target and destroy infected or abnormal cells through the

induction of apoptosis [210]. An elevated CD4/CD8 ratio has been associated with an activated immune state, such as in cases of chronic inflammatory conditions or autoimmune diseases [242-245].

In this study, we noted an increased percentage and number of $CD4^+$ T cells and a decreased percentage of $CD8^+$ T cells in the blood, spleen, MLN, and CLN of BTBR mice compared to the control group. Correspondingly, the $CD4^+/CD8^+$ ratio in all four tissues was higher in the BTBR mice. To our knowledge, this is the first report of alterations to $CD4^+$ and $CD8^+$ T cell levels in the CLN of BTBR mice. However, with regards to the three other tissues analyzed, our data are partially consistent with findings by other groups. Uddin et al. (2020) demonstrated a higher frequency of $CD4^+$ T cells and lower frequency of $CD8^+$ T cells in the peripheral blood and spleens of BTBR mice compared to B6 controls [239]. O'Connor et al. (2021) found that the percentages of $CD4^+$ helper cells were elevated in the MLN and blood of BTBR mice, while $CD8^+$ cell percentages were reduced in the MLN only; in all, the BTBR $CD4^+/CD8^+$ ratio was higher in both the MLN and blood [191]. Yao et al. (2022) also found an increased $CD4^+/CD8^+$ ratio in BTBR spleens [246].

Analysis of $CD4^+$ and $CD8^+$ T cells in human studies of ASD patients has yielded conflicting results. Warren et al. (1986) was one of the first groups to describe T cell alterations in autistic patients (n=31), noting decreased numbers of T lymphocytes and an altered ratio of helper to suppressor (ie. cytotoxic) T cells [247]. In a subsequent comparative study of peripheral blood from 25 ASD patients, Yonk et al. (1990) also noted that autistic subjects had a lower percentage and number of both $CD4^+$ T cells and total lymphocytes compared to their siblings and normal subjects [248]. Conversely,

Ashwood et al. (2011) found no significant differences in the absolute numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T cells of autistic children (n=70) and age- and gender-matched typically developing (TD) controls (n=35) [249]. A more recent study by López-Cacho et al. (2016) analyzing PBMCs from 59 adult ASD patients found an increased percentage of $CD8^+$ T cells and decreased $CD4^+/CD8^+$ ratio compared to control subjects (n=26) [250]. Taken together, the data from BTBR mice studies and clinical ASD studies on $CD4^+$ and $CD8^+$ T cells do not seem to be consistent with each other. Nevertheless, both BTBR mice studies and human have found differences in the $CD4^+/CD8^+$ ratio compared to controls, albeit in opposite directions. The reason behind these inconsistencies is currently unknown and warrants further investigation. The potential of $CD4^+$ and/or $CD8^+$ as biomarkers for ASD remains to be clarified.

There are multiple reports of cytokine abnormalities in autistic patients and preclinical mouse models [226-228]. The exact roles these cytokines play in the development of ASD remain a subject of ongoing investigation. Notably, though, two of the inflammatory cytokines implicated in these alterations, IL-17a and IFN-γ, have been found to mediate behaviors impacted in ASD. Preclinical studies conducted in B6 mice have highlighted the importance of meningeal IL-17a [229] and IFN-γ [230] signaling in the regulation of social behavior and anxiety-like behavior, respectively.

In this current study, we found significantly higher levels of $CD4^+IFN\text{-}\gamma^+$ T cells in the spleens of BTBR mice, which is congruent with findings by Ahmad et al. (2018) [251]. To our knowledge, we are the first to report lowered levels of $CD8^+IFN\text{-}\gamma^+$ T cells in the MLN of BTBR mice. Our data did not reveal a significant strain difference in IL-17a-producing $CD4^+$ or $CD8^+$ T cells in any of the four peripheral tissues tested. This

contrasts previous reports of elevated $CD4^+IL\text{-}17a^+$ T cells in BTBR mouse spleens [251, 252]. The reasons behind the discrepancy is unknown at present.

With regards to ASD patients, one previous clinical study found that proportions of $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ T cells were significantly lower in the peripheral blood of autistic children as compared to healthy controls [253]. These conclusions seem to be consistent with our results that percentages of $CD8^+IFN\text{-}\gamma^+$ T cells are lower in the MLN of BTBR mice. Therefore, both our data in BTBR mice and this precious clinical report point to the potential of $CD8^+IFN\text{-}\gamma^+$ T cells as a biomarker for ASD. However, a very recent study found increased frequencies of $CD4^+IFN\text{-}\gamma^+$ T cells, $CD4^+IL\text{-}17a^+$ T cells, and $CD8^+$ $IFN\text{-}\gamma^+$ T cells [254].

Unlike the better known and more abundant $CD4^+$ and $CD8^+$ T cells, γδ T cells are defined by TCRs composed of γ and δ chains [255]. This unique TCR composition allows them to recognize a broader range of antigens compared to conventional T cells, which primarily recognize peptides presented by major histocompatibility complex (MHC) molecules [255]. Make up about ~5% of all circulating T cells, γδ T cells function independently of MHC-mediated antigen presentation and play a vital role in both innate and adaptive immune responses, as well as autoimmune responses [256]. γδ T cells are primarily found on mucosal surfaces like the intestines, lungs, and vagina, but some have been found to circulate in the blood as well [256]. They have also been found within the meninges of the brain to play important roles in neurobehavior as well as neuroimmune responses [257].

In the current study, we found significantly lower percentages of $TCR\gamma\delta^+$ T cells in the blood, spleen, and MLN of BTBR mice compared to B6 mice. Furthermore, in all

of the peripheral tissues tested, IL-17a-producing and/or IFN-γ-producing TCRγδ$^+$ T cells are lower in the BTBR mice. To our knowledge, this is the first report of differences in TCRγδ$^+$ T cell populations in a preclinical model of ASD.

A recent study performed in children diagnosed with ASD found that the percentage of γδ T cells was significantly elevated, compared to levels in typically developing (TD) children [258]. They noted that γδ T cells, but not αβ T cells, in the peripheral blood were associated with autism [258]. Additionally, it was found that in these autistic patients, there was an increased secretion of IL-17 by circulating γδ T cells, whereas the levels of IFN-γ remained unaffected [258]. While the direction of these results seem to differ from our current findings, both the human study and our murine study found that γδ T cells are associated with ASD. Furthermore, our data support the idea that γδ T cells and their associated cytokine secretion could be potential biomarkers for ASD.

Cytokines are vital signaling molecules primarily produced and released by immune cells, such as T cells, B cells, and macrophages. They help maintain balanced and appropriate immune responses by influencing cell proliferation, differentiation, and activation. These chemical messengers can initiate and propagate inflammatory responses, recruiting immune cells to sites of infection or injury and enhancing the immune system's ability to combat threats. Dysregulation of cytokine signaling and subsequent chronic inflammation can contribute to various pathologies, including autoimmune disorders and neuroinflammatory diseases. The inflammatory hypothesis of ASD suggests immune system dysfunction and abnormal cytokine signaling may cause

neuroinflammation and influence brain development and function, potentially contributing to the behavioral and neurological features of autism.

With regards to clinical studies, many studies looked at plasma levels of the cytokines using ELISA. Some researchers have observed elevated levels of IL-17a in the plasma [259] and whole blood of children with ASD, and the enrichment in IL-17a genes of autistic patients [260]. In addition, increased levels of IFN-γ have been observed in the plasma, brain, and cerebrospinal fluid of ASD patients as well [226, 261]. However, other studies have shown contradictory findings from ASD patients. For example, Onore et al. (2009) reported that IL-17 plasma levels were unchanged in children with ASD compared with TD controls [262]. In addition, the production of IL-17 by PBMCs following in vitro stimulation did not differ between groups [262]. Also, a recent study found that plasma IL-17a was significantly higher in ASD patients compared to healthy controls, but there was no difference in IFN-γ [254]. Therefore, there seems to be no clear clinical consensus on whether the cytokine profiles of IL-17a and IFN-γ are altered in ASD.

For our data, higher levels of $CD4^+IFN\text{-}\gamma^+$ T cells in BTBR spleens are consistent with the inflammatory hypothesis of ASD, which predict higher levels of inflammatory cytokines. However, in most of the lymphoid organs and blood, we did not detect increased intracellular expression of $IFN\text{-}\gamma^+$ or $IL\text{-}17a^+$ in either $CD4^+$ or $CD8^+$ T cells. In fact, a lower percentage of $CD8^+$ $IFN\text{-}\gamma^+$ in BTBR MLN was observed. In addition, lower percentages of IL-17a-producing and/or IFN-γ-producing $TCR\gamma\delta^+$ T cells are found in BTBR mice. This seems conflict with the neuroinflammation hypothesis of ASD and some previous findings showing the IL-17 and IFN-γ levels are higher in tissues from ASD patients. However, it worth mentioning that T cells are not the sole sources of IL-17

and IFN-γ; these cytokines can be secreted by other immune cell types such as natural killer cells and APCs [222-225].

While not entirely in line with the inflammatory hypothesis of ASD, our data may still suggest a role of IL-17a and IFN-γ cytokines in the pathogenesis of autism, nevertheless. For example, our findings of lower percentages of $CD8^+$ IFN-γ^+ and γδTCR^+ IFN-γ^+ T cells in BTBR mice seem to be consistent with previous studies in mice demonstrating that IFN-γ^+ is important for sociability [230], which is one of the core defects of ASD. Thus, our data support the hypothesis that cytokines not only are important for immune responses, but also play crucial roles in animal behavior, such as sociability. In the future, it remains to be studied that if levels of IFN-γ^+ is lower in the brain BTBR mice comparing to B6 mice, and whether this cytokine plays any role in the aberrant sociability of BTBR mice.

Autism is likely influenced by a combination of genetic, environmental, and immunological factors, and the relationship between cytokines and autism is just one piece of this complex puzzle. The role of cytokines in autism is an area of ongoing research. While there is evidence to suggest that immune dysregulation and cytokine imbalances may play a role in some cases of autism, more research is needed to fully understand the mechanisms and their significance in the disorder.

The brain was once considered an immune-privileged organ, separate from the body's immune system. However, research in recent years has challenged this notion, revealing intricate connections between the brain and the immune system. There is an increasing body of evidence pointing to the essential role of microglia, the resident immune cells of the CNS, in ensuring proper brain function, maintaining homeostasis,

and mediating inflammatory signaling. Their expansive involvement in synaptic pruning, neural circuitry, blood-brain barrier integrity, regulating behavior and neuroinflammation is gradually being elucidated [263-265]. In the process, researchers have implicated microglia in various neurological and neurodevelopmental pathologies [266-268].

TREM2 (Triggering Receptor Expressed on Myeloid Cells 2) is a protein primarily expressed on the surface of microglia cells in the brain [269]. TREM2 is a receptor that interacts with various ligands and is involved in modulating microglial function, including phagocytosis of cellular debris, regulation of cytokine production, and modulation of inflammatory responses [269]. Reduced TREM2 expression has been associated with impaired microglial function and increased susceptibility to neurodegenerative diseases [269].

Recently, TREM2 has emerged as a potential regulator of microglial function in ASD [232, 265, 270]. For example, TREM2 knockout mice displayed repetitive behavior and reduced sociability [232]. In addition, absence of TREM2 led to impaired synapse pruning, enhanced excitatory neurotransmission and reduced long-range functional connectivity. Furthermore, in the post-mortem brain of ASD patients, TREM2 levels were negatively correlated with the severity of ASD symptoms [232]. Synaptic pruning is a normal developmental process in which excess or unnecessary synapses in the brain are eliminated, allowing for refinement and optimization of neural circuits. Since it is known that TREM2 is involved in synaptic pruning, it is not surprising that TREM2 may be involved in the pathogenesis of ASD.

In our study, we found no difference in the percentage of $CD11b^{+}CD45^{low}$ microglial cells in whole brains of B6 and BTBR mice. However, the percentage of

microglial cells expressing TREM2 was significantly lower in BTBR mice, compared to controls. These findings suggest potential alterations in microglial activation and TREM2 expression in ASD, which could contribute to neuroinflammatory processes and synaptic dysfunction. In other words, our data suggest that the reduced expression of TREM2 in microglia in BTBR mice may indicate impaired microglial phagocytosis and synaptic pruning. As a result, the known autistic-like core behaviors and dysfunctional neuronal connectivity in the BTBR model could be at least in part due to dysfunctional microglia functions due to low TREM2 expression.

V. Conclusions

In conclusion, in this study we have confirmed previous findings that there is an increase in the percentage of $CD3^+$ T cells and in the ratio of $CD4^+/CD8^+$ T cells in the blood, spleen and MLN of BTBR mice vs. B6 mice. We are the first to show these increases occur in the BTBR CLN as well. We have also demonstrated for the first time that percentages of $\gamma\delta TCR^+$ T cells are lower in BTBR mice, compared to B6 mice. In addition, the percentages of IL-17a- and IFN-γ-expressing γδ T cells are lower in BTBR mice. Furthermore, we have discovered that whole brains of BTBR mice have a lower percentage of TREM2-expressing microglial cells. These data, together with previously published studies, demonstrate that peripheral lymphoid T cell subpopulations, especially γδ T cells and their associated cytokine profiles, as well as TREM2 in the brain, could be potential clinical biomarkers for ASD.

The studies discussed here provide valuable insights into the immune dysregulation observed in a mouse model of idiopathic ASD and offer potential

therapeutic targets. Modulating T cell subsets, such as $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T cells, or altering their cytokine profiles could be explored as therapeutic strategies for restoring immune homeostasis in ASD. Additionally, targeting microglial activation and TREM2 signaling pathways may have therapeutic potential in alleviating neuroinflammation and synaptic dysfunction in ASD.

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EDUCATION:

B.A., Comparative Literature
Brown University, Providence, RI
2011-2015

M.S., Pharmacology and Toxicology
University of Louisville, Louisville, KY
2017-2019

FELLOWSHIPS & GRANTS:

University of Louisville Integrated Programs in Biomedical Sciences Graduate Fellowship
2018-2019

University of Louisville NIEHS T32 Training Program in Environmental Health Sciences
2019

Autism Speaks Predoctoral Fellowship (Grant #11863)
2020-2022

AWARDS & HONORS:

International Cannabinoid Research Society Trainee Award
2018

International Cannabinoid Research Society Braude Foundation Award
2020

International Cannabinoid Research Society Trainee Award
2021

University of Louisville Department of Pharmacology and Toxicology KC Huang Award
2021

University of Louisville Graduate School Dissertation Completion Award
2021

International Cannabinoid Research Society Trainee Award
2022

PROFESSIONAL SOCIETIES:

International Cannabinoid Research Society
2018 – Present

Society for Neuroscience
2019 – Present

American Association for the Advancement of Science
2019 – Present

International Society for Autism Research
2021 – Present

PUBLICATIONS:

Laun AS, **Shrader SH**, Brown KJ, et al. GPR3, GPR6, and GPR12 as novel molecular targets: their biological functions and interaction with cannabidiol, *Acta Pharmacologica Sinica*, 2018 Nov;4(11):e00933. doi: 10.1038/s41401-018-0031-9. (PMID: 29941868)

Laun AS*, **Shrader SH***, Song ZH. Novel inverse agonists for the orphan G protein-coupled receptor 6, *Heliyon*, 2019 Mar;40(3):300-308. doi: 10.1016/j.heliyon.2018.e00933. (PMID: 30480157) (*authors contributed equally to this work)

Shrader SH, Song ZH. Discovery of endogenous inverse agonists for G protein-coupled receptor 6, *Biochem Biophys Res Commun.*, 2020 Feb 19;522(4):1041-1045. doi: 10.1016/j.bbrc.2019.12.004. (PMID: 31818461)

Shrader SH, Tong YG, Duff MB, et al. Involvement of dopamine receptor in the actions of non-psychoactive phytocannabinoids, *Biochem Biophys Res Commun*, 2020 Dec 17;533(4):1366-1370. doi: 10.1016/j.bbrc.2020.10.021. (PMID: 33097185)

Wu J, Chen N, Liu Y, et al. **(Shrader SH 6th author)** Studies of involvement of G-protein coupled receptor-3 in cannabidiol effects on inflammatory responses of mouse primary astrocytes and microglia, *PLoS One*, 2021 May 13;16(5):e0251677. doi: 10.1371/journal.pone.0251677. (PMID: 33984046)

ORAL PRESENTATIONS:

"The Effects of Phyto- and Synthetic Cannabinoids on GPR6 Signaling: Potential Therapeutic Implications" Pharmacology & Toxicology Seminar Series. April 2018. University of Louisville, Louisville, KY.

"Weed, Worms and the Works: The *In Vivo* Effects of Cannabinoids of *C. elegans* and Mice" Pharmacology & Toxicology Seminar Series. July 2019. University of Louisville, Louisville, KY.

"Discovery of Novel Molecular Targets for Endogenous and Phytocannabinoids." Pharmacology & Toxicology Seminar Series. November 2019. University of Louisville, Louisville, KY.

"Exploring Potential Mouse Models of Autism Spectrum Disorder." Pharmacology & Toxicology Seminar Series. October 2020. University of Louisville, Louisville, KY.

"Cannabidiol Alters Social and Repetitive Behaviors in a Model of Idiopathic Autism Spectrum Disorders." June 2021. International Cannabinoid Research Society Annual Symposium. Virtual.

ABSTRACTS & POSTER PRESENTATIONS:

Shrader SH, Laun AS, Song ZH. "The Effects of SR144528 Analogues on GPR3 and GPR6." International Cannabinoid Research Society Annual Symposium. July 2018. Leiden, Netherlands.

Shrader SH, Laun AS, Song ZH. "The Effects of CB2-Selective Antagonist SR144528 and Structural Analogues on GPR3 and GPR6." Research!Louisville. October 2018. University of Louisville, Louisville, KY.

Khalily C, Laun AS, **Shrader SH,** Song ZH. "GPR12-Mediated Alteration of the mTOR Pathway by Phytocannabinoids." Research!Louisville. October 2018. University of Louisville, Louisville, KY.

Shrader SH, Tong YG, Duff MB, et al. "Cannabinoid-Induced Swimming-Induced Paralysis in the Nematode *Caenorhabditis elegans*" Research!Louisville. September 2019. University of Louisville, Louisville, KY.

Duff MB, **Shrader SH,** Song ZH. "Effects of Cannabinoids on Retinal Endothelial Cell Function." Research!Louisville. September 2019. University of Louisville, Louisville, KY.

Shrader SH, Tong YG, Duff MB, et al. "Phytocannabinoid-Induced Swimming-Induced Paralysis in the Nematode *Caenorhabditis elegans*." Society for Neuroscience 2019. October 2019. Chicago, IL.

Shrader SH, Mellen N, Barnes G, Song ZH. "Cannabidiol as a Behavioral Modulator in Autism Spectrum Disorders." International Cannabinoid Research Society Annual Symposium. June 2020. Virtual.

Shrader SH, Mellen N, Barnes G, Song ZH. "Cannabidiol Alters Social and Repetitive Behaviors in a Model of Idiopathic Autism Spectrum Disorders." International Cannabinoid Research Society Annual Symposium. June 2021. Virtual / Jerusalem, Israel.

Sloan LJ, Liang Wei, Duff MB, **Shrader SH,** et al. "CBD Inhibits Angiogenic Processes in Mouse Retinal Microvascular Endothelial Cells." International Cannabinoid Research Society Annual Symposium. June 2021. Virtual / Jerusalem, Israel.

Shrader SH, Mellen N, Barnes G, Song ZH. "Cannabidiol Alters Aberrant Immune Cell Populations in a Model of Idiopathic Autism Spectrum Disorder" International Cannabinoid Research Society Annual Symposium. June 2022. Galway, Ireland.