Gut Microbiome Modulates Microglia Physiology in Homeostatic and Disease States

Thesis by

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ABSTRACT

The gastrointestinal tract (GI) harbors a complex community of ~100 trillion bacteria, fungi, and viruses collectively referred to as the gut microbiome. Through direct and indirect signaling mechanisms, the gut microbiome exerts its effects on almost every organ system, including the brain. Constant, bi-directional communication along the gut-brain axis is required for the normal and healthy development of the host Central Nervous System (CNS). One of the cells in the CNS shaped by microbial-derived cues is microglia, the resident immune cells in the brain. Aberrant microglia activity is a driving force of several neurological diseases in which the gut microbiome plays a role, including Parkinson's disease (PD).

In this thesis, we explore the interplay between gut microbiota signaling and microglia physiology during homeostatic and disease states. We first detail how microbial signaling along the gut-brain axis shapes microglial development and function. Next, we explore how the gut microbiome composition influences microglial activation states in the context of disease. Leveraging a preclinical mouse model of PD, we show that dietary-driven changes to the gut microbiome through the use of prebiotics attenuates motor deficits and α-synuclein aggregation. These effects result from changes in microglial gene expression and activation status. Collectively, these findings have broad implications for the gut microbiome research community and highlight potential for development of microbiome-based therapies for diseases of the brain.

PUBLISHED CONTENT AND CONTRIBUTIONS

Chapter II

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R.A conceived the topics, wrote the review, and designed the figures.

Chapter III

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R.A conceived the project, designed all experiments, executed all experiments, performed all animal work, analyzed data, interpreted the results, and wrote the manuscript.

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ABBREVIATIONS

AD	Alzheimer's disease
ALS	Amytrophic lateral sclerosis
ASD	Autism spectrum disorder
αSyn	Alpha-Synuclein
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CD68	Cluster of differentiation 68
CNS	Central nervous system
Csf1r	Colony stimulating factor 1 receptor
Cx3cr1	CX3 chemokine receptor 1
Ddit4	DNA damage inducible transcript 4
EAE	Experimental autoimmune encephalomyelitis
EMP	Erythro-myeloid progenitor
FFAR2	Free-fatty acid receptor 2
FMT	Fecal microbiota transplant
GF	Germ free
GI	Gastrointestinal
GPCR	G-protein-coupled receptor
Gpr84	G-protein receptor 84
HDAC	Histone deacetylase
IBS	Irritable bowel syndrome
IGF-1	Insulin-like growth factor 1
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4

IL-6	Interleukin 6
Irf8	Interferon regulatory factor 8
Jak3	Janus kinase 3
LCMV	Lymphocytic choriomeningitis
LPS	Lipopolysaccharide
Ly86	Lymphocyte antigen 86
MAMP	Microbial-associated molecular pattern
Mapk8	Mitogen-activated protein kinase 8
MDD	Major depressive disorder
MHCII	Major histocompatibility complex 2
MPTP	Mitochondrial permeability pore
MS	Multiple sclerosis
NGF	Nerve growth factor
P2ry12	Purinergic receptor P2Y12
PCD	Programmed cell death
PD	Parkinson's disease
PET	Positron emission tomography
poly(I:C)	Polyinosinic-polycytidylic acid
Sall1	Spalt like transcription factor 1
SCFA	Short chain fatty acid
SFB	Segmented filamentous bacteria
SIBO	Small intestinal bacterial overgrowth
SPF	Specific-pathogen-free
Stat1	Signal transducer and activator of transcription 1
TLR	Toll-like receptor
Tmem119	Transmembrane protein 119
TNFα	Tumor necrosis factor alpha

Chapter 1

INTRODUCTION

Hippocrates, the father of medicine, postulated that all diseases begin in the gut. We now have a growing body of evidence to substantiate this claim two millennia later. Indeed, residing within the length of our gastrointestinal (GI) tract are trillions of microorganisms (bacteria, fungi, viruses, archaea) collectively referred to as the gut microbiome. The composition and density of microbes vary spatially along the digestive tract. The large intestine harbors the highest bacterial load and is predominantly dominated by the Firmicutes and Bacteroidetes phyla (Donaldson, Lee, and Mazmanian, 2015). Microbial colonization of the host happens immediately at birth, with the microbial flora makeup varying depending on the mode of delivery (vaginal vs Caesarean section) (Moore and Townsend, 2019). In early infancy, the microbiome undergoes many taxonomic changes and eventually stabilizes around three years of age (Derrien, Alvarez, and de Vos, 2019). The composition of the gut microbiome is unique to everyone, akin to a fingerprint. A variety of factors shape an individual's microbial makeup including genetics, age, diet, antibiotic usage, and a variety of other environmental and lifestyle factors (Dieterich, Schink, and Zopf, 2018).

The field of microbiome research has grown at an exponential rate since the turn of the century, from <50 publications in the year 2003 to >10,000 in the present day (Jones, 2013). While there is still much to be uncovered, the studies that have been published to date have been instrumental in highlighting the role of the gut microbiome in health and disease. The gut microbiome plays a critical role in shaping host development and maintaining host health. Conversely, disruptions in the composition of the gut microbiome, referred to as dysbiosis, may be contributing to the onset and progression of many disorders ranging from

metabolic disorders such as obesity and diabetes to neurological conditions including Autism Spectrum Disorders (ASDs) and Parkinson's disease (PD).

Gut microbiome in health

Humans and our gut microbes have coevolved to develop a symbiotic relationship. Human hosts offer microbes an environment to reside and deliver them nutrients, and in exchange, our gut microbiome serves countless functions that are critical for host survival. These include metabolic functions such as nutrient metabolism and absorption, vitamin synthesis, and digestion, as well as immune system development and defense from foreign pathogens (Jandhyala et al., 2015). The gut microbiome also provides structural support, helping to maintain the integrity of the GI tract. The gut microbiome exerts its effects on host physiology through a variety of mechanisms. This includes the production of metabolites such as short chain fatty acids (SCFAs), bile acids, and amino acids, that can act locally or systemically to influence downstream signaling pathways (Krautkramer, Fan, and Bäckhed, 2020).

Mouse models have served as powerful research tools for uncovering the various functions of the gut microbiome and shedding light on the mechanism by which gut microbes influence host physiology. One mouse model commonly used in proof-of-concept experiments is germ-free (GF) mice. GF mice lack microbes on and in their body and are housed in isolators to maintain their sterile status. Using GF mice, researchers can evaluate how the absence of microbial colonization and signaling impacts various organ systems. An alternative experimental paradigm for studying gut-host interactions is antibiotic

(ABX) treated-mice. ABX treatment enables researchers to study how microbial signaling impacts the host during various developmental windows and time points. Moreover, specific ABX cocktails allow selective depletion of subsets of bacteria (Kennedy, King, and Baldridge, 2018). Studies using GF and ABX-treated mice have highlighted the importance of gut microbiome signaling on host immune system development and responses, metabolism and digestion, and organ system development (Kennedy, King, and Baldridge, 2018).

Gut-Brain axis

In addition to metabolic and immune-related functions, input from the gut microbiome is critical for normal brain development. Indeed, constant bi-directional communication between the gut microbiome and the brain along the gut-brain axis has been shown to facilitate the development and functioning of the host Central Nervous System (CNS). Signaling along the gut-brain axis encompasses physical (vagal transmission) and chemical (neurotransmitter/neuropeptides, microbial metabolites, immune cytokines) modes of communication (Morais, Schreiber, and Mazmanian, 2020). Gut-to-brain signaling can influence host nervous system development, mood, behavior, cognition, and satiety while brain-to-gut signals impact GI permeability, motility, and intestinal permeability (Carabotti et al., 2015; Morais, Schreiber, and Mazmanian, 2020).

Absence of microbial signaling has profound effects on CNS development and behavior. At the molecular and structural level, GF mice demonstrate altered neurogenesis, microglial physiology, neurotransmitter profiles, stress hormone levels, and reduced blood-

brain barrier (BBB) integrity (Luczynski et al., 2016). Molecular deficits culminate in changes at the behavioral level, as GF mice display altered anxiety, sociability, self-grooming, feeding behavior, locomotion, and cognitive abilities (although specific changes vary across studies) (Luczynski et al., 2016).

Gut microbiome and microglia

With 70-80% of immune cells residing in the gut, it is unsurprising that the gut microbiome shapes the development of the innate and adaptive immune system in the gut and periphery (Wiertsema et al., 2021). These findings, along with increased evidence of gut-brain communication, has prompted investigation into the link between the gut microbiome and the brain's immune system. Microglia are the resident innate, immune cells in the CNS, comprising ~80% of immune cells in the brain (Morimoto and Nakajima, 2019). Microglia play important roles in neural circuitry development, neuronal health, and injury response and repair (Wolf, Boddeke, and Kettenmann, 2017). Researchers believed microglia to be shielded from peripheral signals by the BBB for many decades; however, recent evidence is challenging this notion. In addition to the constant stream of information from neighboring cells, microglia receive and process input from peripheral signals, including the gut microbiome (Erny et al., 2015). These findings, discussed in detail in Chapter 2 of this thesis, paved the way for a burgeoning area of research aiming the better understanding how the gut microbiome influences microglia physiology during homeostatic and disease states.

Gut microbiome in disease

Increasing evidence over the past few years has illustrated how the gut microbiome is a double-edged sword. While the gut microbiome is critical for several facets of host development and health, perturbations to the microbiome composition (i.e. dysbiosis) may be causing and/or contributing to a variety of diseases. Disturbances to the gut microbiome can include expansion of pathogenic species, depletion of health-promoting bacteria, or a combination of both (Wilkins, Monga, and Miller, 2019). The precise root of dysbiosis is not known but may be attributed to antibiotic use, infection, or a highly-processed and low-fiber diet (Martinez et al., 2021). To date, the most compelling evidence for gut microbiome involvement in disease pathology is for GI-related conditions including inflammatory bowel disease (IBD) and *Clostridium difficile* infection (Gorkiewicz and Moschen 2018). However, a role for the gut microbiome extends beyond diseases of the GI tract. Indeed, preclinical and clinical evidence implicates the gut microbiome in metabolic conditions (diabetes, obesity), several types of cancer, and cardiovascular disease (Buford, 2017). Additionally, dysregulated signaling along the gut-brain axis may be a contributing force of neurodegenerative (PD, Alzheimer's disease (AD)) and neuropsychiatric disorders (depression, schizophrenia) (Morais, Schreiber, and Mazmanian, 2020). Interestingly, aberrant microglial activity has been demonstrated in a majority of these conditions, suggesting a potential role for gut-microglia crosstalk in the onset and progression of neurological disorders (Wolf, Boddeke, and Kettenmann, 2017).

Human studies investigating the role of the gut microbiome in disease often involve profiling the composition of the gut microbiome in healthy and disease populations and

searching for robust differences between the two. Variations in gut microbiome composition have been reported in patient populations of PD (Keshavarzian et al., 2015), type 2 diabetes (Das et al., 2021), and schizophrenia (Li et al., 2020), among others. Although these comparative studies are instrumental in identifying potential microbes or groups of microbes that may contributing to disease, they remain correlative and descriptive in nature.

Preclinical mouse models are a powerful tool to elucidate the role of the gut microbiome during disease states. Mouse models allow researchers to perform more mechanistic studies that establish a causal role for the microbiome in disease pathology. A causal role for the gut microbiome has been shown in several GF mouse studies, wherein GF mice have an improved disease outcome relative to their specific-pathogen-free (SPF) counterparts. The absence of a gut microbiome reduces motor deficits, neuroinflammation, and protein aggregation in a GF mouse model of PD (Sampson et al., 2016). Similar effects have been observed in GF models of AD (Colombo et al., 2021; Harach et al., 2017) and diet-induced obesity (Bäckhed et al., 2007). GF mice can also be used to interrogate the effects of one specific bacterial strain (monocolonization) or a group of bacteria (Round and Palm, 2018). These studies allow investigators to identify "probiotics," bacterial strain(s) that promote the growth of healthy bacteria in the gut and produce a desirable health outcome.

A caveat to mouse studies is that their gut microbiome composition and GI architecture differs from humans. Researchers have attempted to overcome this limitation using what the field refers to as "humanized" mice. Humanized mice are generated by transplanting

fecal samples from humans into GF mice, generating a mouse model with a human microbiome. These studies are useful for establishing causality for the microbiome in various disease states. Remarkably, several studies have revealed that key hallmarks of disease are transferable via the microbiome in humanized mouse models of schizophrenia (Zheng et al., 2019), PD (Sampson et al., 2016), autism (Sharon et al., 2019), and multiple sclerosis (Cekanaviciute et al., 2017) .

Gut microbiome therapeutic modalities

Given our growing knowledge of the gut microbiome's involvement in a variety of conditions, efforts to develop gut microbiome targeted therapeutics are underway. To date, therapies targeting the gut microbiome include three main modalities: prebiotics, probiotics, and fecal microbiota transplant (FMT) (Fong, Li, and Yu, 2020). Prebiotics are dietary fibers defined as nondigestible food substrates that support the growth of healthy bacteria in the gut (Davani-Davari et al., 2019). Interest in the application of prebiotics as a therapeutic tool has been catalyzed by an increasing number of studies linking Western diet consumption (low fiber, highly processed food) with increased risk of autoimmune disease, depression, and PD (Jacka et al., 2010; Manzel et al., 2014; Mischley, Lau, and Bennett, 2017). Probiotics also have promising therapeutic potential through attenuating colonic inflammation, preventing apoptosis, and suppressing growth of pathogenic bugs (Fong, Li, and Yu, 2020). In 2020 over 1000 clinical trials using probiotics were registered for the treatment of >700 different conditions (Dronkers, Ouwehand, and Rijkers, 2020). Lastly, FMT involves the transplantation of a healthy individual's stool to an individual

with a microbiome-related illness. While this procedure accompanies risks, FMT has been proven successful in the treatment of C. difficile infection (Rohlke and Stollman, 2012).

Thesis overview

The work presented in this thesis is at the intersection of neuroscience, immunology, and microbiology. In this thesis, we explore the interactions between the gut microbiome and microglia, the resident innate immune cells of the CNS. Chapter 2 is a published review article in which we explore the literature in depth and describe how the gut microbiome shapes microglia development and physiology during health and disease. Chapter 2 illustrates the various conduits of communication that make up the gut-brain-axis and explores the link between the gut microbiome and microglia in neuropsychiatric and neurodegenerative conditions. In Chapter 3, we identify and characterize a prebiotic (high-fiber) diet that attenuates motor deficits and α-synuclein (αSyn) aggregation in the Thy1-α-synuclein-overexpressing (ASO) mouse model of PD. Additionally, we explore how prebiotic intervention changes the composition of the gut microbiome and levels of bacterial-derived metabolites. To better understand how dietary intervention shapes the CNS, we dive into characterization of microglia physiology. We apply immunohistochemistry and single-cell RNA sequencing to probe changes to microglia morphology and gene expression in wild type and ASO mice on control vs. prebiotic diets. Lastly, in Chapter 4 we end with a discussion on the current state of the gut microbiome research field, discuss novel microbiome-targeted therapeutic strategies, and end with our projections on how the field will evolve over the next 10-20 years.

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Chapter 2

MICROBIOME-MICROGLIA CONNECTIONS VIA THE GUT-BRAIN AXIS

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Abstract

Microglia, the resident immune cells in the brain, are essential for modulating neurogenesis, influencing synaptic remodeling, and regulating neuroinflammation by surveying the brain microenvironment. Microglial dysfunction has been implicated in the onset and progression of several neurodevelopmental and neurodegenerative diseases; however, the multitude of factors and signals influencing microglial activity have not been fully elucidated. Microglia not only respond to local signals within the brain but also receive input from the periphery, including the gastrointestinal (GI) tract. Recent preclinical findings suggest that the gut microbiome plays a pivotal role in regulating microglial maturation and function, and altered microbial community composition has been reported in neurological disorders with known microglial involvement in humans. Collectively, these findings suggest that bidirectional crosstalk between the gut and the brain may influence disease pathogenesis. Herein, we discuss recent studies showing a role for the gut microbiome in modulating microglial development and function in homeostatic and disease conditions and highlight possible future research to develop novel microbial treatments for disorders of the brain.

Introduction

Microglia are tissue-resident macrophages that make up ~ 5-15% of total brain cells and have several well-defined functions in the central nervous system (CNS) (Pelvig et al., 2008). During early development, microglia actively regulate neuronal cell numbers and synaptic refinement, ultimately shaping neural circuitry (Sierra et al., 2010; Paolicelli et al., 2011; Wynn et al., 2013). To sustain brain homeostasis, microglia constantly survey their microenvironment through the dynamic extension and retraction of their processes (Davalos et al., 2005; Nimmerjahn et al., 2005). Upon sensing signals of infection or injury, microglia transition from a homeostatic surveillance state to an activated state, facilitating antimicrobial or tissue repair programs that restore homeostasis (Saijo and Glass, 2011).

In addition to important roles in brain development and homeostasis, recent genetic studies provide evidence that microglia contribute to the pathogenesis of several neurodegenerative and neurodevelopmental disorders (Salter and Stevens, 2017). However, environmental factors and mechanisms shaping the developmental, homeostatic, and pathogenic program of microglia remain poorly understood. Within the CNS, microglial activity is governed in part by cytokines and chemokines, neurotransmitters, and other molecules that regulate signaling pathways that influence various brain functions (Xavier et al., 2014). Once thought to be shielded from the circulatory system by the blood–brain barrier (BBB), microglial activity is now known to be influenced by factors originating outside the CNS, including the gut. Sophisticated crosstalk between the CNS and the gut microbiome (known as the gut–brain axis) is

critical for several facets of CNS physiology, including microglial development and function (Mayer et al., 2015; Fung et al., 2017). Recent studies provide important insights into the role of gut microbiota in microglial maturation, identity, and function, both in steady state conditions and in diseases associated with elevated microglial activation. These findings have sparked a new field in microbiology focused on identifying and mapping direct and indirect interactions between the gut microbiota and microglia.

In this review, we will highlight mechanisms by which the gut–brain axis regulates microglial identity and function during development and aging. We then discuss gut–brain communication pathways and how perturbations in the healthy gut microbiota (i.e., dysbiosis) could potentially lead to microglial dysfunction. Finally, we will highlight possible interactions of the microbiome and microglia in the context of neurodevelopmental and neurodegenerative disorders exemplified by autism spectrum disorder (ASD) and Parkinson's disease (PD), respectively.

Microglia during development and adulthood

Microglia maturation

Microglia were first discovered in the early 20th century by Pío del Río-Hortega, who pioneered exploration of microglial morphology and function (Sierra et al., 2016). Until recently, the origins and precise lineage of microglia have been subjects of significant debate in the biomedical research community. The analogous function and structure between microglia and macrophages inspired the hypothesis that these cells share a common lineage. However, the advent of new methods to study cellular lineages,

including genetic tracing, transgenics, and fate-mapping analyses, defined distinct developmental trajectories and ontogenies between these two cell populations (Ginhoux et al., 2010).

Microglial development is thought to be precisely orchestrated by an intrinsic genetic program and environmental cues (Fig. 1). This process begins in the yolk sac around embryonic day 7.5 (E7.5) as microglia emerge from erythromyeloid progenitor cells, which are hematopoietic precursor cells of the mesoderm (Alliot et al., 1999; Ginhoux et al., 2010; Kierdorf et al., 2013; Gomez Perdiguero et al., 2015; Hoeffel et al., 2015; Sheng et al., 2015). The maturation and differentiation of erythromyeloid progenitors into microglia within the yolk sac requires several transcription factors, including RUNX1, JUN, PU.1, and IRF8, the expression of which coincides with that of microglial markers, including CX3CR1, CD11b, and F4/80 (Ginhoux et al., 2010; Matcovitch-Natan et al., 2016). At E8.5, microglia become mobile and begin to migrate from the embryonic yolk sac to the brain. This process of brain colonization precedes the formation of the BBB, which eventually shields microglia from potentially toxic peripheral influences throughout both fetal development and adulthood (Obermeier et al., 2013). Once in the brain, microglia are broadly distributed at varying densities and maintain a stable rate of proliferation depending on the stage of host development (Askew et al., 2017). The capacity for microglia to self-renew in their local environment, independent of hematopoietic progenitor cells circulating the bloodstream, is a defining feature of these innate immune cells (Gomez Perdiguero et al., 2015).

Early microglia identity and function

Transcriptomic studies suggest that after populating the brain, microglia undergo a stepwise maturation program in parallel to brain development, from early microglia (until E14) to premicroglia (E14 to the first weeks after birth) and finally adult microglia (Fig. 1; Matcovitch-Natan et al., 2016). Early microglia and premicroglia gene expression signatures are associated with cellular development, growth, and proliferation, whereas genes enriched in adult microglia are associated with immune signaling pathways (Matcovitch-Natan et al., 2016; Thion et al., 2018).

In addition to innate immune cell functions that are characteristic of all tissue-resident macrophages, recent evidence has elucidated additional developmental and homeostatic functions of microglia that are specific to the nervous system. During the early stages of brain development, early microglia and premicroglia phagocytose excess neurons and release neurotrophic and neurotoxic factors, thereby controlling the relative ratio of neurogenesis to apoptosis to ensure that numbers of neurons are maintained within a defined range (Sierra et al., 2010; Cunningham et al., 2013). In addition to dictating neuronal density, microglia supply a steady stream of neurotrophic factors (such as nerve growth factor [NGF], brain-derived neurotrophic factor [BDNF], and insulin-like growth factor 1 [IGF-1]) that promote neuronal survival and differentiation of neural progenitors (Gomes et al., 2013; Ueno et al., 2013).

Microglia also play a role in establishing and shaping neural circuitry during postnatal stages of development, which has implications for cognitive function and social behavior

(Paolicelli et al., 2011). Synaptic remodeling resulting in the removal of excess synapses eliminates redundancies in neural circuitry and improves efficiency in neural crosstalk. In a remarkable parallel to macrophage recognition of pathogens, this process has been shown to depend on several complement proteins (C1q and C3) that tag extraneous synapses for microglial engulfment (Stevens et al., 2007; Schafer et al., 2012). Furthermore, high-resolution microscopy has confirmed a physical, albeit transient, interaction between microglia and synapses on neighboring neurons (Tremblay et al., 2010).

Adult microglia identity and function

In mice, microglia transition to an adult phenotype a few weeks after birth. The adult microglia transcriptome overlaps with, but is distinct from, other tissue-resident macrophages and is characterized by expression of microglial-specific markers, including *Sall1*, *P2ry12*, *Gpr84*, and *Tmem119* (Hickman et al., 2013; Butovsky et al., 2014; Gosselin et al., 2014; Matcovitch-Natan et al., 2016). While microglia are heavily involved in shaping the neuronal and synaptic landscape during early development, they are more actively involved in homeostasis and immune surveillance during later developmental stages and adulthood, as suggested by up-regulation of genes involved in immune regulation (Matcovitch-Natan et al., 2016). Systematic analyses of human microglial gene expression from postmortem and surgical tissues indicate broad similarities between human and mouse microglial gene expression but also significant differences, particularly with regard to expression of genes associated with the

pathogenesis of neurodevelopmental and neurodegenerative diseases (Galatro et al., 2017; Gosselin et al., 2017).

Adult microglia are a morphologically dynamic population of cells; they display a wide spectrum of structural and molecular phenotypes that reflect the status of their extracellular environment at a given time. Depending on the surrounding microenvironment, microglia can exist in a "surveying" or "active" state. Under steady-state conditions, surveying microglia have a ramified morphology with a small cell body and many long extended processes that are used to continuously scan and assess the health of cells in close proximity—a process critical for maintaining homeostasis in the absence of pathology (Nimmerjahn et al., 2005; Torres-Platas et al., 2014). Upon insult to brain tissue, microglia swiftly activate, retracting their processes and transitioning to an amoeboid morphology with an enlarged cell body (Nimmerjahn et al., 2005; Torres-Platas et al., 2014). Depending on the nature of the insult, microglia can initiate pro- or anti-inflammatory signaling cascades. Activation of pro-inflammatory signaling pathways causes microglia to release pro-inflammatory cytokines (e.g., IL-6, IL-12, IL-1β, and TNF-α) and reactive species (e.g., nitric oxide and reactive oxygen species) into their surrounding environment to suppress and fight off invading pathogens (Franco and Fernández-Suárez, 2015; Tang and Le, 2016). Conversely, activation of anti-inflammatory pathways allows microglia to mitigate and repair damage caused by the initial immune stimulus and the pro-inflammatory response. Activation of these pathways triggers release of anti-inflammatory cytokines (e.g., IL-4, IL-10, and TGF-β) and neurotrophic factors that prevent development of chronic inflammation and allow

microglia to maintain their neuroprotective and wound-healing properties (Franco and Fernández-Suárez, 2015). Maintaining tight control over microglial activation states is critical for CNS health, given the risk of pathology that is associated with heightened neuroinflammation.

In addition to their innate immune and homeostatic functions, microglia have been implicated in the pathogenesis of a broad spectrum of neurodegenerative and behavioral diseases, including PD, Alzheimer's disease (AD), multiple sclerosis (MS), schizophrenia, and ASD (Vargas et al., 2005; Hirsch et al., 2012; Frick et al., 2013; Bilimoria and Stevens, 2015). In each case, there is evidence of immune activation, suggesting a role for microglia-driven inflammation as an etiologic factor (Perry and Holmes, 2014; Norden et al., 2015). Of particular interest, recent genetic studies have identified a large number of coding and noncoding risk alleles for neurodegenerative and behavioral diseases that affect genes highly or preferentially expressed in microglia (Lambert et al., 2013; Welter et al., 2014). Thus far, the risk alleles that have been identified are largely nonoverlapping across diseases and affect genes involved in diverse cellular processes, implying complex and poorly understood mechanisms linking microglia to neurodegeneration and behavioral disorders.

Gut-brain axis influences microglia development and function

Until recently, the microbiome did not spark much attention among neuroscientists. However, recent work characterizing the extensive communication between the gut and

the brain has demonstrated an active role for gut bacteria in governing several aspects of CNS physiology.

The ~100 trillion microorganisms that reside in the digestive tract, and the wide assortment of metabolites they produce, are critical for maintaining health (Lloyd-Price et al., 2016). Within this complex community in humans are >1,000 bacterial species (Frank and Pace, 2008). Initial microbial colonization of the gut happens at birth and is heavily influenced by the mode of delivery (cesarean section versus vaginal birth; Dominguez-Bello et al., 2010; Rodríguez et al., 2015). In the first few years of life, the gut microbiota is relatively less diverse and less stable compared with that of adults, with an abundance of Proteobacteria and Actinobacteria (Palmer et al., 2007; Rodríguez et al., 2015). By the age of 5, the gut microbiota stabilizes and begins to resemble that of an adult, with members of Bacteroidetes and Firmicutes becoming most abundant (Eckburg et al., 2005).

Advances in sequencing technologies and bioinformatic tools to study the gut microbiome have contributed to a greater appreciation of its diversity, plasticity, and paramount role in a multitude of physiological functions. Along with preserving the integrity of the intestinal–epithelial barrier along the gastrointestinal (GI) tract, gut bacteria are critical for the development and maturation of the host's innate and adaptive immune systems, nutrient absorption, host metabolism, and protection against foreign invaders (Hooper et al., 2001; Bäckhed et al., 2004; Geuking et al., 2011; Round et al.,

2011). Indeed, the functions of the gut microbiota extend beyond the physical borders of the digestive tract in which they reside. The diverse repertoire of metabolites and signaling molecules produced by gut bacteria enter the systemic circulation, facilitating the molecular crosstalk between host and microbes throughout the body (Martinez et al., 2017).

Communication between gut microbes and the CNS is mediated by a combination of immune, enteric, and neural pathways that provide physical and chemical connections between the CNS and the periphery, and several experimental paradigms have been used to demonstrate that gut microbes influence many facets of CNS physiology (Mayer et al., 2015; Fung et al., 2017; Yoo and Mazmanian, 2017). Germ-free (GF; i.e., lacking all commensal and pathogenic microbes) mice, antibiotics, fecal microbiota transplant (FMT), and pre-/probiotics have demonstrated a role for gut bacteria in neurotransmitter signaling, synaptic plasticity, myelination, and neurogenesis (Diaz Heijtz et al., 2011; Neufeld et al., 2011; Ogbonnaya et al., 2015; Hoban et al., 2016). Additionally, the absence of gut microbes causes deficits in cognition and social interaction, further supporting the role of gut microbes in higher-order brain functioning (Neufeld et al., 2011; Luczynski et al., 2016).

The gut microbiota affects various cells in the CNS, including microglia. Indeed, recent studies have demonstrated that microglia are sensitive to factors produced by the gut microbiota. Striking differences in the structure and function of microglia derived from specific pathogen–free (SPF) and GF mice have been observed, both at the genetic and

morphological level (Erny et al., 2015). Since then, new work has defined additional factors and pathways by which gut microbes influence microglial maturation and function within the CNS.

Maternal microbiota shapes prenatal microglial maturation and function

While the womb is likely a sterile environment, new findings suggests that signals from maternal gut microbes may shape the developmental trajectory of fetal microglia close to the time of birth (Thion et al., 2018). At E14.5, embryonic microglia from offspring of GF dams display minor differences in gene expression compared with their SPF counterparts, whereas microglia collected close to birth (E18.5) demonstrate marked differences in gene expression, chromatin accessibility, morphology, and regional distribution (Thion et al., 2018). For example, microglia from E18.5 embryos from GF mothers show an increased density in various brain regions, with increased numbers of microglia exhibiting a ramified morphology, indicative of a resting state. Altered microglial morphology and density, as well as attenuated inflammatory responses, are also observed in offspring of GF dams immediately after birth, a time when microglia typically exhibit an activated phenotype (Castillo-Ruiz et al., 2018).

Interestingly, sex-related differences have been observed with regard to the influence of the maternal gut microbiota on microglial function in offspring. In male offspring of GF dams, disruption of the transcriptomic profile and morphology of microglia was greatest during the embryonic phase of development, and differentially regulated genes were mostly associated with metabolic and translational pathways (Thion et al., 2018). In female offspring, by contrast, disruption was most notable in adults, and differentially

expressed genes were primarily involved in immune and transcriptional signaling (Thion et al., 2018). This suggests that input of maternal gut microbes may have a greater impact on early microglia and premicroglia in male offspring than in females. These trends might help to explain the inherent functional differences in microglia from intact male versus female mice, as well as the sex variations in incidence rates of several neurological disorders (Schwarz et al., 2012; Hanamsagar et al., 2015). The heterogeneity of microglial behavior in response to both intrinsic and extrinsic factors provides further evidence of their complexity, with the gut microbiome representing a key contributing factor in microglial development and function.

Diverse gut microbiota is a prerequisite for adult microglial maturation and function

Consistent with patterns observed prenatally, microglia from adult GF mice, lacking constant postnatal input from gut microbiota, show distinct differences in density and morphology compared with those derived from SPF mice (Erny et al., 2015). Morphological variations in microglia from adult GF mice include increased cell volume, dendrite length, segment number, and branch points. Microglia from adult GF mice also display differential expression of genes associated with microglial maturation, including down-regulation of genes that regulate cell activation and immune system defense pathways, such as *Mapk8*, *IL-1α*, *Ly86*, *Jak3*, and *Stat1*, all of which are normally highly expressed in adult microglia (Fig. 1; Erny et al., 2015; Matcovitch-Natan et al., 2016). Concurrently, genes highly expressed in microglia during early developmental stages that promote cell proliferation and survival, including *Csf1r* and *Ddit4*, are aberrantly up-regulated in microglia isolated from adult GF mice.

The immature gene expression profile resulting from the absence of gut microbiota contributes to the inability of microglia to properly respond to immunostimulants such as LPS and the lymphocytic choriomeningitis virus, both of which failed to elicit an appropriate activation response in microglia from GF mice (Erny et al., 2015). The dampened immune response to both LPS and lymphocytic choriomeningitis virus included a relative decrease in microglial expression of genes encoding pro-inflammatory cytokines (e.g., IL-1β, IL-6, and TNF-α) and a reduction in microglia with an activated, amoeboid morphology. These findings suggest that the gut microbiota is likely imperative for an adequate microglia-mediated immune response against pathogens invading the CNS. Taken together, the gene expression profile and behavior of microglia from GF mice or under microbiota-depleted conditions is reminiscent of an immature microglial phenotype, indicating that input from gut microbiota is required for microglia to progress to later stages of cellular maturation and adequately fulfill their role in immune surveillance.

To examine the extent to which microbial colonization influences microglial physiology, Erny et al. (2015) characterized microglia from mice co-colonized with *Bacteroides distasonis*, *Lactobacillus salivarius*, and *Clostridium* cluster XIV. Microglia from mice with such a limited microbial complexity displayed a genetic signature and morphology similar to that observed in microglia from GF mice. However, recolonization of those mice with a more diverse microbial community facilitated the transition to a mature microglial phenotype typically found in adult SPF animals. Thus,

the presence of a complex and diverse microbial community, rather than exposure to gut bacteria per se, appears to be a prerequisite for proper microglial development and function.

Investigations into the temporal window for microbe-mediated regulation of microglial maturation has revealed the need for constant input from a diverse gut microbiota. This claim is supported by the conversion of microglia from adult SPF mice into an immature phenotype following antibiotic administration to deplete the microbiota. With the exception of indiscernible differences in microglial density, microglia isolated from antibiotic-treated SPF mice exhibit a microglial gene expression profile and morphology nearly identical to those derived from GF mice (Erny et al., 2015; Thion et al., 2018). These findings suggest that microglia are highly sensitive to perturbations in the gut microbial community during adulthood and require continual input from a complex gut microbiota to maintain homeostasis in the adult.

Gut-brain communication pathways: Vagal transmission and systemic circulation

Despite the many unanswered questions regarding the intersection between gut microbiota and microglial physiology, there is evidence that pathways that collectively integrate the gut–brain axis influence microglial function in both homeostatic and disease conditions (Fig. 2). Gut–brain communication may influence microglia via two routes: the vagus nerve and the circulatory system.

The vagus nerve

Thousands of sensory and motor fibers from the vagus nerve connect the gut and the brainstem and serve as a conduit for neural signals. Communication through the vagus nerve is essential for signals mediating satiety, stress, and mood, and these signals are governed by changes in enteric neuron activity and the behavior of gut microbes (Goehler et al., 2005; Forsythe et al., 2014; Browning et al., 2017). Given their close physical proximity, symbiotic and pathogenic gut bacteria can directly interact with and activate the vagus nerve, thereby exerting effects upstream to the CNS. Oral inoculation with the pathogen *Campylobacter jejuni* or intraduodenal injection of the probiotic strain *Lactobacillus johnsonii* are sufficient to induce activation of vagal sensory neurons innervating the GI tract, as well as neurons in the brainstem (Goehler et al., 2005; Tanida et al., 2005). Additionally, metabolites and neuroactive substances produced by microbes activate chemoreceptors located at vagal nerve endings (Hara et al., 1999; Raybould, 2010). Indeed, the anxiolytic effects of administration of the probiotic species *Lactobacillus rhamnosus* and *Bifidobacterium longum* is absent in vagotomized mice, providing strong evidence that gut–vagal nerve interactions regulate social behavior (Wang et al., 2002; Bercik et al., 2011; Bravo et al., 2011).

Communication between intestinal microbes and vagal afferents also appears to influence microglia and the level of inflammation in the CNS (Forsythe et al., 2014). In addition to interacting with gut microbes, vagal nerves interact extensively with different components of the peripheral immune system, continuously monitoring the inflammatory state of the gut (Borovikova et al., 2000; Miao et al., 2004). Upon sensing a change in

inflammation, such as increased production of pro-inflammatory cytokines, vagal afferents relay this information to the CNS and can ultimately influence the level of neuroinflammation (Goehler et al., 1999). Concurrently, vagal efferent nerves relay information about the immune status of the brain back to the gut, with increased CNS inflammation feeding back to inhibit further release of peripheral pro-inflammatory cytokines through acetylcholine-mediated signaling (Wang et al., 2003; Goehler et al., 2005). Effective vagal nerve signaling is critical for sending appropriate signals to microglia in order to modulate levels of neuroinflammation. Electrical stimulation of the vagus nerve in the presence of an immune challenge in the periphery has downstream effects on microglial behavior, including up-regulation of anti-inflammatory pathways in the brain (Frasch et al., 2016; Meneses et al., 2016; Kaczmarczyk et al., 2017). Vagus nerve stimulation combined with LPS challenge has also been reported to decrease microglial production of the pro-inflammatory cytokines IL-6, IL-1β, and TNFα in the brain, an effect no longer observed following vagotomy (Meneses et al., 2016). These findings support the role of the vagus nerve as a physical conduit between gut microbial activity and neuroinflammation.

Regulation of intestinal barrier and peripheral immune response

The presence of bacteria along the GI tract is critical for the maintenance of the intestinal barrier, which facilitates the exchange of nutrients, water, and electrolytes and prevents the passage of harmful substances and pathogens from the intestinal lumen into the bloodstream (Jakobsson et al., 2015). By altering expression levels of tight junction proteins along the epithelial wall, and thus the level of bacterial infiltration in the

mucosal layer, the gut microbiota can fine-tune the level of intestinal permeability (Karczewski et al., 2010; Alaish et al., 2013). The regulation of the intestinal barrier by gut microbiota shapes their role as mediators of the intestinal and peripheral immune response. Decreased strength of the intestinal barrier due to dysbiosis and other factors permits entry of pathogenic, immune-stimulating, and neuroactive substances into the systemic circulation (Kelly et al., 2015). Once in the circulation, these substances activate a pro-inflammatory immune response mediated by peripheral T cells and macrophages and compromise the integrity of the BBB (Rochfort et al., 2014). Increased circulation of BBB-permeable pro-inflammatory cytokines and neurotoxic compounds may contribute to heightened microglial activation and production of pro-inflammatory cytokines in the brain (Qin et al., 2008; Riazi et al., 2008).

Along with affecting the level of permeability along the intestinal tract, gut microbiota can influence the state of peripheral inflammation through interactions with nearby immune cells. Approximately 70–80% of immune cells in the human body are found in the gut, allowing for direct gut–immune cell interactions (Vighi et al., 2008). When microbe-associated molecular patterns produced by pathogenic invaders bind to pattern recognition receptors, such as TLRs, on host cells, they influence the production of both pro- and anti-inflammatory cytokines (Fung et al., 2017). The circulation and subsequent entry of these cytokines into the brain acts locally on CNS cells, including microglia, that express the appropriate cytokine receptors, thereby influencing the state of inflammation in the brain. Indeed, increased intestinal inflammation driven by either LPS or bacterial infection correlates with elevated levels of microglial activation and release of pro-

inflammatory cytokines (Riazi et al., 2008; Henry et al., 2009). These studies provide further confirmation of the intimate link between peripheral inflammation, influenced in part by the gut microbiota, and microglial activation and neuroinflammation.

Bacterial-derived neuroactive substances

Microbe-derived neuroactive metabolites are additional contributors to gut–brain crosstalk. Circulation of microbe-derived neurotransmitters, including acetylcholine (*Lactobacillus*), GABA (*Bifidobacteria* and *Lactobacillus*), and serotonin (*Enterococcus* and *Streptococcus*), can potentially influence microglial activation through direct and indirect means (Komatsuzaki et al., 2005; Yano et al., 2015; Pokusaeva et al., 2017). Studies have demonstrated that 90% of serotonin required for the regulation of mood, behavior, sleep, and several other functions within the CNS and GI tract is produced in the gut. Binding of serotonin to 5-HT receptors on microglia induces release of cytokine-carrying exosomes, providing another mechanism for gut-induced modulation of neuroinflammation (Glebov et al., 2015). Another microbial metabolite that influences microglia activity is tryptophan, a serotonin precursor. Metabolism of tryptophan by activated microglia produces the neurotoxin quinolinic acid, an N-methyl-D-aspartate agonist implicated in several neurological conditions including Huntington's disease and depression (Feng et al., 2017). In a recent study using the experimental autoimmune encephalomyelitis (EAE) mouse model of MS, peripheral metabolism of dietary tryptophan by the gut microbiota was shown to generate metabolites that dampen the ability of microglia to induce pro-inflammatory responses in astrocytes, thereby ameliorating disease (Rothhammer et al., 2018). These findings provide further

confirmation of the role of gut microbiota in influencing behavioral and physiological functions previously thought to be exclusively controlled by local factors in the brain.

Short-chain fatty acids (SCFAs) are metabolic byproducts of bacterial dietary fiber fermentation that can enter the systemic circulation and cross the BBB (Conn et al., 1983). Among the most abundantly produced SCFAs are acetic acid, propionic acid, and butyric acid, which collectively make up ~95% of SCFAs synthesized in the gut (Cook and Sellin, 1998). SCFAs can exert physiological effects in the CNS via two primary mechanisms: activation of G protein–coupled receptors (GPCRs) expressed in the liver, spleen, and large intestine and inhibition of histone deacetylases (Kimura et al., 2011; Tan et al., 2014b). SCFAs have been shown to activate sympathetic nervous system activity and alleviate intestinal inflammation, and altered SCFA production has been demonstrated in a variety of neuropathologies such as PD and ASD (Smith et al., 2013; MacFabe, 2015; Unger et al., 2016).

More recently, the effects of SCFAs have been extended to microglia. Supplementation of drinking water with a mixture of three primary SCFAs (acetic acid, propionic acid, and butyric acid) rescued the immature genetic and morphological phenotype of microglia from GF mice (Erny et al., 2015). However, the exact SCFA signaling pathways that modulate microglial maturation have yet to be fully elucidated. SPF mice lacking the free fatty acid receptor 2 (FFAR2), a GPCR required for SCFA signaling in the gut, exhibited a microglial phenotype similar to that observed in GF mice (Erny et al., 2015). The

absence of FFAR2 expression on microglia suggests that SCFAs may influence microglial maturation through signals that originate in the GI tract. However, exactly how this signal propagates to the CNS and governs microglial behavior is poorly understood. In addition to GPCR signaling, the ability of SCFAs to permeate the BBB and infiltrate the CNS suggests potential direct influences on microglia. Indeed, treatment of microglial cells in vitro with various SCFAs, including valproic acid and butyric acid, elevates levels of acetylation of histone H3 (Chen et al., 2007). This suggests that the ability of SCFAs to influence microglial behavior in vivo might occur through a combination of GPCR signaling and histone deacetylase inhibition. Together, these studies highlight the potential of gut-derived metabolites to enter the systemic circulation and exert their effects on microglia in the CNS.

Microglial dysfunction and microbial dysbiosis in disease

Given their wide spectrum of physiological functions and myriad roles in development and homeostasis, microglia are believed to be involved in the pathogenesis of several neurodevelopmental and neurodegenerative disorders. However, the factors and signals within the brain environment and the periphery that modulate microglial function and response during disease are not fully understood. The recent increase in research into gut–brain communication has created a new narrative for how the microbiome may shape microglial identity and function and how the microbiome, via microglia, may modulate the pathogenesis of neurological diseases. Accordingly, understanding the gut–brain axis will provide the foundation for potential novel therapeutic approaches in the years ahead.

Because of our increased awareness of the gut–brain axis, it has become clear that various neurological diseases, once thought to originate exclusively in the brain, are influenced by peripheral factors. The possible involvement of the microbiota in neurodevelopmental and neurodegenerative diseases stems from two primary observations. First is the critical role of gut-derived factors in regulating microglial function in the healthy state, which suggests that signals originating from the gut microbiota might also drive a pathogenic microglial phenotype that promotes disease. Second, dysbiosis is observed in several neurological conditions in which microglial dysfunction is thought to be a contributing factor to disease development, including ASD and PD (Table 1; Hsiao et al., 2013; Sampson et al., 2016). This dysbiosis is potentially sufficient to markedly disrupt microglial function and subsequently facilitate disease pathogenesis. Here, we discuss an emerging role for the gut–brain axis in ASD and PD, where most work has been done to date.

ASD

Children with ASD present with a wide range of cognitive and behavioral symptoms, including delayed language development, impaired social and communication skills, and repetitive behavior, depending on where they lie on the spectrum of disease severity (Plauche Johnson, 2004). In addition, symptoms of gut dysfunction such as irritable bowel syndrome, chronic diarrhea, and/or constipation are found in 23–70% of patients with ASD (Chaidez et al., 2014). The high prevalence of GI comorbidities among ASD patients and the correlation between the level of GI distress and severity of ASD symptoms has prompted studies investigating whether the development and/or

progression of ASD has microbial origins. Cross-sectional studies comparing the gut microbiota composition between healthy and ASD individuals have revealed an altered microbiota profile in patients with ASD, with several studies reporting increased levels of *Clostridium* and *Lactobacillu*s along with elevated levels of SCFAs, including propionic and butyric acid (Wang et al., 2012, 2013; De Angelis et al., 2013; Kang et al., 2013). However, given the small sample size in these pilot studies, further studies with larger cohorts are warranted.

While the etiology of ASD is complex and incompletely determined, microglia may influence the course of the disease. A collective consequence of microglial dysfunction is stunted neuronal development and immature brain circuitry, which could ultimately manifest in the ASD behavioral phenotypes. Postmortem analysis of brain tissue collected from ASD patients show perturbations in microglial immune surveillance and synaptic remodeling, with evidence for heightened microglial activation, including increased expression of MHC II, elevated levels of pro- and anti-inflammatory cytokines, and increased microglial density (Vargas et al., 2005; Morgan et al., 2010; Voineagu et al., 2011; Gupta et al., 2014; Lee et al., 2017). Impaired synaptic remodeling by microglia might contribute to the increased density of dendritic spines and excitatory synapses found in the brains of patients with ASD (Martínez-Cerdeño, 2017). Findings from animal models also support a possible role for microglia in ASD. Mice lacking the fractalkine receptor *Cx3cr1* demonstrate a temporary reduction in the number of microglia during early postnatal development, as well as increased synaptic density, immature brain circuitry, and signal transmission deficits that persist into adulthood

(Paolicelli et al., 2011; Zhan et al., 2014). Abnormal neural circuitry due to the absence of microglia during a critical window of brain development in these mice is associated with behavioral deficits similar to those seen in individuals with ASD (Zhan et al., 2014).

Other preclinical models have provided insight into the role of the microbiota and microglia in driving the pathology of ASD (Needham et al., 2018). The development of the maternal immune activation (MIA) model was motivated by the increased incidence rate of ASD in children whose mothers suffered from a severe infection during certain stages of pregnancy. In the MIA model, offspring of mice injected with polyinosinic-polycytidylic acid (poly(I:C)), a synthetic viral mimic that activates TLR3, demonstrate core symptoms of ASD, including repetitive behaviors, communication deficits, and decreased sociability (Malkova et al., 2012). These MIA offspring also exhibit increased intestinal permeability and intestinal inflammation, two GI symptoms commonly found in children with ASD (Hsiao et al., 2013; Chaidez et al., 2014). Provision of IL-17 promoting segmented filamentous bacteria to MIA mothers further enhances behavioral abnormities in MIA offspring (Kim et al., 2017). While the gut microbiota contributes to ASD symptomatology in both mice and humans, it also has protective effects. Similar to its ameliorative effect in colitis, oral administration of *Bacteroides fragilis* to offspring of MIA mice at weaning rescues the integrity of the intestinal barrier, reduces anxiety, and improves communication and repetitive behaviors (Hsiao et al., 2013). Similarly, provision of *Lactobacillus reuteri* to offspring of dams fed a high-fat diet attenuates their social deficits, further supporting a role for the gut microbiota in influencing outcomes of neurodevelopmental disorders (Buffington et al., 2016).

While commensal symbiotic bacteria are critical for proper microglial maturation, induction of infection in pregnant dams can have disruptive effects on the progression of microglial development in offspring (Pratt et al., 2013; Matcovitch-Natan et al., 2016; Mattei et al., 2017). Microglia from young MIA offspring show increased expression of inflammation-related genes typically associated with an adult microglial phenotype and down-regulation of genes expressed during earlier developmental stages (e.g., Pu.1 and *Irf8*), suggesting that maternal inflammation disrupts the timeline of normal microglial maturation (Matcovitch-Natan et al., 2016). Adult MIA offspring exhibit heightened microglial activation, as noted by increased levels of IL-6 and TNF-α (Pratt et al., 2013; Mattei et al., 2017). However, the differences in the microglial transcriptome seen in offspring from MIA versus healthy dams decline as mice reach adulthood. These findings indicate that the effects of maternal inflammation on microglial development and behavior in offspring may be restricted to a narrow developmental window, after which microglia revert to a relatively normal phenotype in adulthood. While the MIA model has limitations in terms of replicating the complex symptomatology of ASD, the model faithfully recapitulates abnormalities in both microglial behavior and GI function that are frequently observed in patients. Replication of microbiome effects on microglia in additional animal models would help strengthen the gut–brain link to neurodevelopmental disorders.

Studies of ASD and other neurodevelopmental disorders provide evidence for a potential pathogenic role for both dysbiosis and microglial dysfunction, and suggests that microglia may link early-life dysbiosis to long-lasting neurological abnormalities.

Perturbations in the composition of gut microbes during early developmental stages due to maternal infection, mode of delivery, antibiotic use, and early-age infections increase an individual's predisposition to atypical behavioral patterns. Given the requirement for constant input from the gut microbiota for normal microglial development and function, it is plausible that microbial effects on neural development and behavior may occur through changes in microglial activity (Erny et al., 2015; Thion et al., 2018). New evidence to further support this link will provide a greater appreciation for the role of a healthy gut microbiome in normal microglial and cognitive development.

PD

PD is the second most common neurodegenerative disorder and is defined by the presence of motor symptoms including bradykinesia, rigidity, and resting tremor (Postuma et al., 2015). Pathological hallmarks of PD include death of dopaminergic neurons of the substantia nigra pars compacta and intraneuronal accumulation of the α-synuclein protein (Goedert et al., 2013). A complex interplay of genetic and environmental factors is thought to drive the pathogenesis of PD, eventually leading to mitochondrial and autophagic dysfunction (Shulman et al., 2011).

One prevailing theory to explain synucleinopathies is that progressive alterations in the microenvironment of microglia, such as increased deposition of α-synuclein, can change microglial behavior and function (Zhang et al., 2005; Su et al., 2008). These changes may ultimately trigger a neuropathogenic microglial phenotype that facilitates and accelerates PD pathology. Heightened microglial activation results in the release of pro-

inflammatory cytokines (e.g., IL-6 and TNF-α) and a variety of other neurotoxic compounds into their immediate extracellular environment (Zhang et al., 2005; Kim and Joh, 2006). This in turn could compromise neuronal function and eventually lead to synaptic degeneration and neuronal death. The sustained activation of microglia due to external cues from both misfolded α-synuclein and damaged neurons likely instigates a cycle of chronic inflammation that further perpetuates the death of dopaminergic neurons and accelerates progression of the disease (Zhang et al., 2005; Kim and Joh, 2006).

Although PD is predominantly classified as a brain disorder affecting neurons of the nigrostriatal pathway, some believe that pathology originates in the gut. According to Braak's hypothesis, aggregation of α-synuclein spreads from the enteric nervous system to the brain via the vagus nerve in cases of sporadic PD (Braak et al., 2004; Rietdijk et al., 2017). Evidence for this theory is supported by both preclinical and clinical evidence demonstrating the presence of α-synuclein deposits in enteric neurons of the gut before the detection of misfolded α-synuclein in the CNS (Braak et al., 2004; Bencsik et al., 2014). While this pattern of α-synuclein spreading is not observed in all cases of sporadic PD, vagotomy may be associated with reduced risk of developing PD in humans, potentially implicating peripheral influences on disease development (Svensson et al., 2015; Liu et al., 2017).

Given the possible crosstalk between the gut microbiota and microglia, the composition of intestinal bacteria may modulate disease pathogenesis. Nonmotor symptoms, including chronic constipation and GI distress, precede motor symptoms in up to 80% of PD

patients (O'Sullivan et al., 2008; Poirier et al., 2016; Unger et al., 2016). Moreover, differences in the gut microbiota composition, bacterial load, and levels of bacterial metabolites have been observed in patients with PD when compared with healthy individuals (Hill-Burns et al., 2017). Studies show altered abundance of certain bacterial strains in patients with PD, changes that may correlate with severity of motor deficits (Scheperjans et al., 2015; Mertsalmi et al., 2017). While interindividual variation is high, PD patients often exhibit increased levels of Enterobacteriaceae and decreased levels of Bacteroidetes and Prevotellaceae (Keshavarzian et al., 2015; Unger et al., 2016). Small intestinal bacterial overgrowth is an additional defining disease feature in 25–54.5% of PD patients (Fasano et al., 2013; Tan et al., 2014a). The shift in microbial communities may contribute to the elevated levels of peripheral inflammation and intestinal permeability that are frequently seen in PD patients and might drive misfolding of α-synuclein in the gut and its retrograde propagation to the CNS.

Support for the notion that the microbiota may drive the pathogenesis of PD was provided by studies in GF mice that overexpress human α-synuclein. These mice display reduced motor deficits, GI dysfunction, and microglial activation when compared with mice with an intact gut microbiota (Sampson et al., 2016). This observation suggests that the gut microbiota, along with genetic predisposition, may be required for disease onset and/or progression. Feeding these GF transgenic mice a mixture of three SCFAs up-regulated microglial activation and induced motor deficits similar to those observed in colonized animals (Sampson et al., 2016). In another recent study, FMT from healthy mice into mice injected with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a

toxin-induced model of PD, attenuated microglial activation and motor deficits and decreased SCFA levels, suggesting that active signals from the gut microbiota may influence PD outcomes (Sun et al., 2018). Interestingly, a recent study showed that overall fecal SCFA concentrations were reduced in patients with PD compared with controls, while some specific SCFAs were relatively increased (Unger et al., 2016). The current uncertainties in the role of SCFAs in various PD models may stem from concentration- and region-dependent effects of SCFAs on host physiology. Despite these preliminary findings, precisely how the gut microbiota and microbial metabolites influence motor symptoms and neuroinflammation in PD remains poorly understood.

The presence of both microbial dysbiosis and microglial dysfunction has been characterized in behavioral (schizophrenia and depression), neuroinflammatory (MS), and neurodegenerative (AD and amyotrophic lateral sclerosis [ALS]) disorders (Table 1). Whether gut microbiota directly or indirectly affect microglia in these conditions remains largely unknown. Gradual changes in microbiota composition have been observed as normal features of aging, including changes in microbial resilience, stability, and diversity, which are features that occur alongside changes in microglial physiology with age (Zapata and Quagliarello, 2015; Buford, 2017). Similar to neurodevelopmental disorders, this trend suggests that microbiota-driven changes in microglial behavior might have a larger role in the onset or progression of neurodegenerative disorders than previously thought (Buford, 2017). Studies investigating changes in microbiome composition and microglial function in the healthy and diseased brain over time will

provide additional insights into the nature of gut–brain interactions during the aging process.

Future directions and challenges

Tremendous progress has been made recently in elucidating and characterizing the distinct components and signals of the gut–brain axis. However, the studies to date likely only represent an initial glimpse into the functional interplay between the gut microbiome, microglia, and neurodevelopmental and neurodegenerative disorders. The advent of new tools, such as advanced next-generation sequencing methods used to study and characterize the microbiome and microglia, will facilitate further identification and characterization of mechanisms by which gut microbiota influence microglial maturation and function (Bennett et al., 2016). For example, the application of single-cell sequencing to study microglia has also paved the way for potential identification of unique microglial subsets with neuroprotective roles in the context of neurodegenerative disease (Keren-Shaul et al., 2017). These studies have shifted the narrative from the notion of exclusively pathogenic microglia to one of a more nuanced mixture of microglial subsets, including some with neuroprotective properties, enabling a greater appreciation of the multifaceted role microglia might play in driving neuropathological phenotypes and potentially accelerating the development of microglia-targeted therapies.

One of the outstanding questions is how changes in the gut microbiome might lead to an altered microglial phenotype and eventually to impaired brain homeostasis. It is unclear whether the observed disease-associated alterations in microbial communities are a cause

or consequence of altered brain function and whether interventions targeting the microbiome can restore microglial function and lead to beneficial effects in neurodevelopmental and neurodegenerative diseases. Signals originating from the gut microbiota and transmitted to the brain have the potential to alleviate or exacerbate disease pathogenesis, changes that may operate through gut-mediated changes in microglial behavior. Thus, continued exploration of the intersection of microbiology, immunology, and neurobiology holds immense therapeutic promise. Several different microbiome-targeted approaches, including prebiotic, probiotic, and FMT strategies, have shown promising results for a variety of GI conditions in preclinical and clinical models and could be extended to pathologies involving microglia in the near future (Fond et al., 2015; Winek et al., 2016). Further investment in gut–brain axis research may catalyze the potential of harnessing the gut microbiome for development of innovative, noninvasive, and effective therapeutic strategies for disorders of the brain.

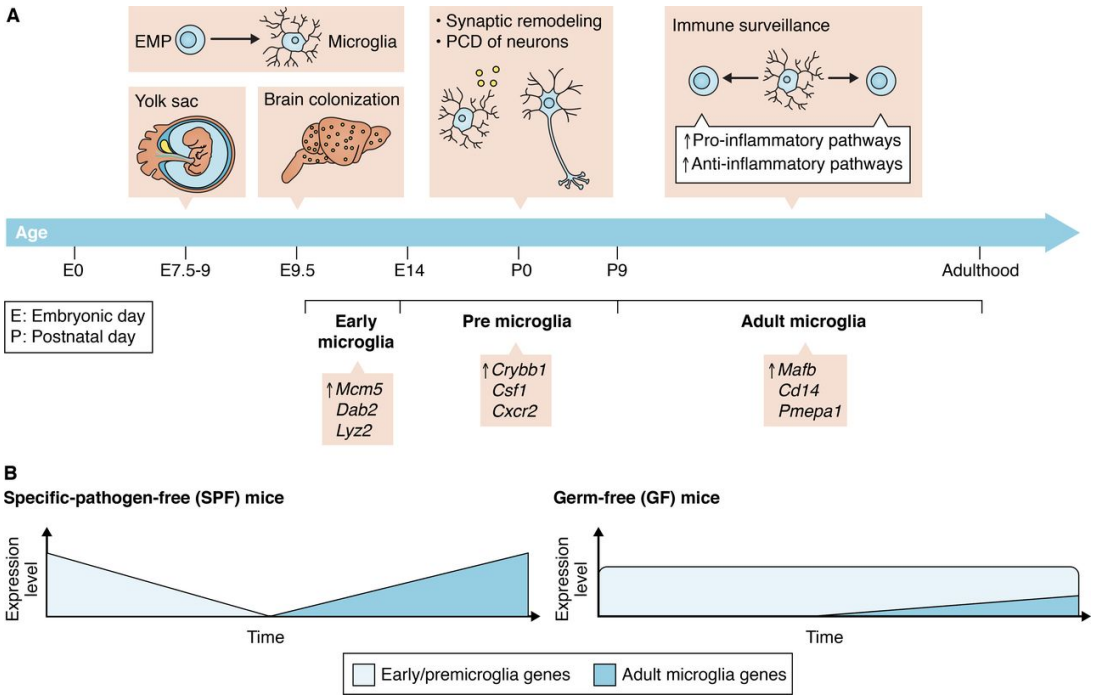

Figure 1: Gut microbiota influences microglial development and maturation. **(A)** Microglial maturation states can be described in three primary phases: early, pre-, and adult microglia. Each phase of development can be defined by expression of a subset of genes that correspond to a core set of microglial functions. Early and premicroglia have two main functions during early brain development: synaptic remodeling and subsequent shaping of neural circuitry and regulating the number of neurons through mechanisms of programmed cell death (PCD). A few weeks after birth, microglia transition to the "adult microglia" stage, in which they constantly survey their immediate surroundings and actively maintain homeostatic conditions. In the presence of tissue damage or an immune stimulus, microglia activate pro- and anti-inflammatory signaling cascades to clear pathogens and repair tissue damage to restore brain health. Recent evidence suggests that prenatal and postnatal inputs from the gut microbiota are critical for microglial maturation and function. **(B)** In SPF mice, a diverse gut microbiota promotes microglial development and maturation. Microglial development appears arrested in GF mice, as supported by high expression of genes characteristic of early and premicroglia in microglia from adult GF mice. This arrest in microglial maturation impedes their ability to initiate a sufficient immune response during infection. EMP, erythromyeloid progenitor.

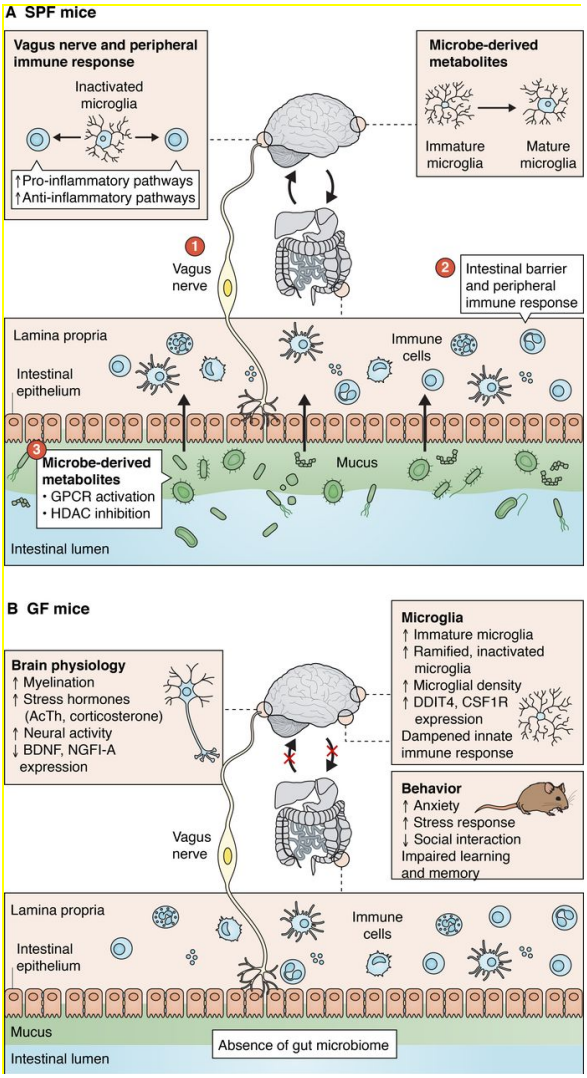

Figure 2: Gut-brain communication pathways. Communication between the gut microbiota and the CNS encompasses several conduits along neural, enteric, and immune pathways. **(A)** Proper microglial maturation and behavior is dependent on crosstalk along the gut–brain axis. Information about the state of peripheral inflammation and GI health is received in the CNS via vagal afferents that innervate the GI tract and can influence microglial activation and neuroinflammation. Fine-tuning of the intestinal barrier by gut microbiota and their interactions with gut immune cells modulates peripheral inflammation and can trigger downstream inflammatory responses in the CNS. BBB-permeable bacterial metabolites, including SCFAs, modulate microglial maturation through mechanisms that are yet to be determined. **(B)** The absence of gut microbes in GF mice confers a variety of physiological abnormalities in neural and microglial behavior in the CNS, resulting in heightened anxiety, stress, hyperactivity, and other behavioral symptoms. BDNF, brain-derived neurotrophic factor; HDAC, histone deacetylase.

Table 1: Neuropathologies characterized by both microglial dysfunction and microbial dysbiosis.

Neuropat-hology	Categoriza-tion	Hallmarks of Microglial Dysfunction	Hallmarks of Microbial Dysbiosis	References
Autism Spectrum Disorder (ASD)	Neurodevel-opmental	• Elevated microglial activation and release of pro-inflammatory cytokines in several brain regions. • Synaptic and neural circuitry dysfunction found in postmortem brain tissue from individuals with ASD. • Mice lacking microglia during early stages of postnatal development demonstrate cognitive and behavioral hallmarks reminiscent of ASD, in addition to abnormal neuronal signaling.	• 23-70% of individuals with ASD report gastrointestinal symptoms (i.e., constipation, abdominal bloating). • Increased *Clostridium* and *Lactobacillus* and decreased *Bacteroidetes* and *Bifidobacterium* found in fecal samples collected from children with ASD. • Decreased SCFA levels in ASD patients compared to healthy controls. • *M*onocolonization of GF mice with *Bacteroides fragilis* attenuates cognitive and gastrointestinal defects in mice.	(De Angelis et al. 2013; Hsiao et al. 2013; Kang et al. 2013; Wang et al. 2013; De Rubeis et al. 2014; Gupta et al. 2014; Hsiao 2014; Zhan et al. 2014; Koyama and Ikegaya 2015; Martínez-Cerdeño 2017)
Schizophr-enia	Neuropsych-iatric	• Increased microglial activity observed in positron emission tomography (PET) scan of schizophrenic patients. • Elevated pro-inflammatory cytokine release (IL-2, IL-6, IL-8, TNF-α) and neuroinflammation in CNS. • Elevated microglial density in temporal cortex of schizophrenic patients. • Microglia-mediated disruptions in white matter structure and volume in prefrontal cortex. • Abnormal synaptic remodeling by microglia disrupts neural circuitry in schizophrenic patients due to increased expression of complement proteins C3 and C4.	• Risk factors for schizophrenia involve disruptions to gut microbial community including: maternal infection, premature delivery, C-section delivery, and young-age viral infection. • High levels of colitis and GI dysfunction in schizophrenic patients. • GF and MIA mice display schizophrenic-like behaviors (i.e., decreased sociability, anhedonia). • Oropharyngeal microbiota of schizophrenic patients is less diverse than controls and enriched in *Lactobacilli, Bifidobacterium,* and *Eubacterium* and depleted in *Neisseria* and *Haemophilus.* • Schizophrenic patients demonstrate dysregulation of several metabolic pathways regulated by the gut microbiota.	(West et al. 2006; Severance et al. 2010; Shaw 2010; Diaz Heijtz et al. 2011; Miller et al. 2011; Monji et al. 2013; Na et al. 2014; Yolken and Dickerson 2014; Bloomfield et al. 2015; Castro-Nallar et al. 2015; Hercher et al. 2014; Reisinger et al. 2015; Severance et al. 2015; Sekar et al. 2016; van Kesteren et al. 2017)

Major Depressive Disorder (MDD)	Neuropsych-iatric	• Postmortem analysis of human brain tissue reveals elevated microglial activation and density in MDD patients. • Increased microglial secretion of exosomes carrying pro-inflammatory cytokines in individuals with MDD. • Chronic stress, a partial contributor to/risk factor for depression, is attributed to increased microglia-driven neuroinflammation. • Precise role of heightened neuroinflammation in brain in MDD remains poorly understood.	• High concurrence between gastrointestinal disorders, such as IBS, and MDD. • Probiotic supplementation of *Lactobacillus case*i improved mood in patients with depression. • Mouse model of MDD exhibiting high levels of stress has increased levels of *Clostridium* and reduced levels of *Lactobacillus* and *Bacteroides.*	(Benton et al. 2007; Dinan and Cryan 2013; Bailey et al. 2011; Brites and Fernandes 2015; Yirmiya et al. 2015; Marin et al. 2017)
Parkinson's Disease (PD)	Neurodegen-erative	• High levels of microglial activation found in substantia nigra in brain tissue from PD patients. • PET scans from 11 PD patients reveals widespread microglial activation in basal ganglia and temporal and frontal cortex that exceed the level of activation found in healthy controls. • α-synuclein aggregates trigger microglial activation in substantia nigra. • Microglial release of pro-inflammatory cytokines and neurotoxic factors is a contributing factor to dopaminergic cell death. • Heightened microglial activation observed in several Parkinsonian-like transgenic mice (α-synuclein over-expression) and toxin-induced mouse models (MPTP, 6-OHDA, rotenone).	• >80% of PD patients report gastrointestinal dysfunction (i.e.,. increased intestinal permeability, constipation, nausea) 10-20 years prior to onset of motor symptoms. • Microbiota of PD patients demonstrate increased levels of *Enterobacteriaceae* and decreased levels of *Bacteroidetes* and *Prevotellaceae*. • Concentrations of SCFAs (acetate, propionate, butyrate) lower in fecal samples collected from PD patients. • SIBO observed in 25-54.5% of patients. • Misfolding and aggregation of α-synuclein may begin in enteric neurons that innervate the gut. • GF mice overexpressing α-synuclein demonstrate attenuated motor and gastrointestinal symptoms compared to their SPF counterparts.	(McGeer et al. 1988; Akiyama and McGeer 1989; Kim and Joh 2006; Gerhard et al. 2006; Watson et al. 2012; Fasano et al. 2013; Tan et al. 2014; Keshavarzian et al. 2015; Scheperjans et al. 2015; Machado et al. 2016; Poirier et al. 2016; Sampson et al. 2016; Unger et al. 2016; Zhang et al. 2017)
Alzheimer's Disease (AD)	Neurodegen-erative	• PET scans and postmortem analysis of brain tissue from AD patients reveals elevated microglial activation correlating with severity of disease in several brain regions (hippocampus, entorhinal cortex, parietal cortex). • Microglia found to drive propagation of tau protein. • Microglia aggregation surrounding amyloid beta plaques. • Neurodegeneration occurs partially in response to microglia-driven chronic inflammation • Neuroprotective microglia subtype recently identified operating through TREM2-mediated signaling pathway. • Complement protein, C1q, involved in mediating microglial synaptic remodeling, is upregulated in AD mouse models.	• The absence of a microbiota in a GF mouse model of AD reduces aggregation of amyloid beta, microglial activation, and neuroinflammation. • Reduction of microbial diversity following antibiotic administration reduced amyloid beta pathology and microglial activation in AD mice. • Microbiota of APPPS1 transgenic mice have a higher Bacteroidetes/Firmicutes ratio compared to WT mice along with reduced levels of *Verrucomicrobia*. • *In vitro* administration of several SCFAs (valeric acid, propionic acid, and butyric acid) obstructs aggregation of amyloid beta protein.	(Xiang et al. 2006; Shie et al. 2009; Koenigsknecht-Talboo et al. 2008; Ferrarelli 2015; Hamelin et al. 2016; Hong et al. 2016; Minter et al. 2016; Harach et al. 2017; Keren-Shaul et al. 2017; Ho et al. 2018)

Amyotrophic lateral sclerosis (ALS)	Neurodegen-erative	• PET scans from ALS patients demonstrate high levels of microglial activation in motor cortex and prefrontal cortex. • Microglial release of pro-inflammatory cytokines and neurotoxic factors (TNF-α, IL-1β) increases as disease progresses. • Microglia expressing mutated Cu,Zn superoxide dismutase (SOD1), a familial ALS gene, accelerates loss of motor neurons and disease progression while wild type microglia conferred neuroprotective effects. • The neuroprotective role of anti-inflammatory microglia found in early stages of ALS is lost as increased levels of pro-inflammatory microglial activity drives neurodegeneration. • Secretion of mutated SOD1 protein into extracellular space triggers microglial dysfunction and activation.	• Small pilot study finds decreased microbial diversity in five ALS patients characterized by intestinal inflammation, low Firmicutes/Bacteroidetes ratio, and low SCFA levels. • G93 ALS mice expressing mutant SOD1 protein have lower expression of intestinal epithelial tight junction proteins and subsequent disruption to intestinal barrier. • G93 mice have a varying gut microbiota composition compared to healthy control mice with reduced levels of *Escherichia coli*, *Fermicus,* and butyrate-producing bacteria. • Drinking water supplemented with the SCFA, butyrate, improved intestinal barrier function and life expectancy in G93 ALS mouse model.	(Turner et al. 2004; Beers et al. 2006; Zhao et al. 2010; Gerber et al. 2012; Zhao et al. 2013; Wu et al. 2015; Geloso et al. 2017; Rowin et al. 2017; Zhang et al. 2017)
Multiple Sclerosis (MS)	Autoimmune/ Neurodegen-erative	• Co-localization of activated microglia and areas of demyelination and inflammatory lesion in MS patients and experimental autoimmune encephalomyelitis (EAE) mice. • Activated microglia produce reactive oxygen species that contribute to oxidative stress and heightened neuronal injury, neurodegeneration, and demyelination. • Inhibiting microglial activation prevented onset of EAE in mice and decreased presence of CNS lesions. • Microglia-mediated remyelination is impaired in MS patients. • Activation of microglia during early stage of disease facilitates recruitment of T cells from periphery. • Subsets of microglia with activated TNFR2 and TREM2 signaling demonstrate a neuroprotective role in EAE mice. • Whether microglial-driven neuroinflammation is a cause or a consequence of neurodegeneration in MS remains unclear.	• Patients with MS have high levels of intestinal permeability. • High concurrence of inflammatory bowel disease and MS. • Dysbiosis found in MS patients (n=20) characterized by depleted levels of *Bacteroides* and *Prevotella* and enriched levels of *Bifidobacterium* and *Streptococcus* compared to healthy controls. • Patients (n=31) with MS have an altered microbiota composition compared to age-and gender-matched controls, with increased levels of *Pseudomonas* and *Mycoplana*. • Monocolonization of GF mice with different species enriched in MS patients (*A. muciniphila*, *P. distasonis*) influenced differentiation of regulatory T cells and lymphocytes. • Development and severity of EAE is lower in GF mice and antibiotic-treated mice compared to SPF mice as shown by an attenuated release of pro-inflammatory cytokines.	(Yacyshyn et al. 1996; Benveniste 1997; Heppner et al. 2005; Sun et al. 2006; Piccio et al. 2007; Yokote et al. 2008; Frischer et al. 2009; Napoli and Neumann 2010; Lee et al. 2011; Vogel et al. 2013; Miyake et al. 2015; Cekanaviciute et al. 2017; Gao et al. 2017; Kosmidou et al. 2017; Luo et al. 2017)

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Chapter 3

A PREBIOTIC DIET MODULATES MICROGLIA RESPONSE AND MOTOR DEFICITS IN α-SYNUCLEIN OVEREXPRESSING MICE

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This chapter will be submitted for publication.

Abstract

Parkinson's disease (PD) is a movement disorder characterized by neuroinflammation, α-synuclein pathology, and neurodegeneration. Interactions between the gut microbiota and immune cells in the brain, namely microglia, have been proposed to impact the pathophysiology of neurodegenerative disorders, including PD. Levels of short-chain fatty acids (SCFAs), produced by gut microbiota from dietary fiber, are altered in human PD and in mouse models, and these bacterial metabolites impact microglial activation states in mice. We therefore investigated whether a fiber-rich diet influences microglial function in α-synuclein overexpressing (ASO) mice, a preclinical model with PD-like symptoms and pathology. We find that, compared to control diets, a prebiotic high-fiber diet attenuates motor deficits and reduces α-synuclein aggregation in key brain regions, and reduces microglial response to α-synuclein compared to control diets. Concomitantly, the gut microbiome of ASO mice adopts more protective features following prebiotic treatment. Single-cell RNA-seq analysis of microglia from the striatum and substantia nigra uncovers increased pro-inflammatory signaling and reduced homeostatic responses in ASO mice compared to WT counterparts. These pathogenic features are reversed by prebiotic feeding, which also promotes expansion of protective disease-associated macrophage (DAM) subsets. Notably, depletion of microglia using a CSF1R inhibitor eliminates the beneficial effects of prebiotics. We thus uncover a microglia-dependent interaction between diet and motor performance in mice, findings that may have implications for neuroinflammation and PD.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder in the United States. It currently affects 1-2% of the population over the age of 65, and the incidence rate is projected to double between 2015 and 2040, mainly due to lifestyle factors and increased lifespan (Dorsey et al., 2018). Despite advances in clinical and basic research, safe and effective PD treatments are still lacking. Clinical features of PD include slowed movement, muscle rigidity, resting tremors, and postural instability. These symptoms result from death of dopaminergic neurons of the nigrostriatal pathway regulating motor function (Poewe et al., 2017). Abnormal aggregation of the neuronal protein α-synuclein (αSyn) promotes disruptions in multiple cellular processes that contribute to neurodegeneration, including mitochondrial dysfunction, oxidative stress, proteasomal impairment, autophagy deficits, and neuroinflammation (Poewe et al., 2017).

Although PD is predominantly classified as a brain disorder, 70-80% of patients experience gastrointestinal (GI) symptoms, mainly constipation but also abdominal pain and increased intestinal permeability, in the prodromal stage of the disease (Forsyth et al., 2011; Yang et al., 2019). Braak's hypothesis postulated nearly 20 years ago that αSyn aggregation may start at peripheral environmental interfaces, like the GI tract or olfactory bulb, and eventually reach the brain stem, substantia nigra, and neocortex via the vagus nerve (Braak et al. 2003). Increasing evidence has corroborated a gut-to-brain spread of αSyn pathology in rodents (S. Kim et al., 2019; B. Liu et al., 2017; Svensson et al., 2015). To date, several studies have detected differences in gut microbiome composition between PD patients and healthy controls, as well as overgrowth of bacteria in the small

bowel (Çamcı and Oğuz, 2016; Keshavarzian et al., 2015; Scheperjans et al., 2015; A. H. Tan et al., 2014). While there are specific differences between datasets, emerging trends include a decreased abundance of health-promoting bacteria and an increased abundance of pro-inflammatory pathogenic bacteria in the PD microbiome. Altering the microbiome in PD-mimicking α-synuclein overexpressing (ASO) mice modulates brain pathology and motor performance (Sampson et al., 2016), and some gut bacterial species have been shown to accelerate disease in PD mouse models (Choi et al., 2018; Sampson et al., 2020). Additionally, antibiotic treatment improves motor symptoms in several mouse models of PD (Cui et al., 2022; Pu et al., 2019; Sampson et al., 2016).

There are multiple conduits of communication between the gut and the brain, including vagal pathways, neuroimmune and neuroendocrine interactions, and microbial metabolite signaling (Morais, Schreiber, and Mazmanian, 2020). Short-chain fatty acids (SCFAs) are metabolic byproducts of bacterial fiber fermentation in the large intestine. Levels of the three most abundant SCFAs, acetate, propionate and butyrate, are significantly reduced in fecal samples from PD patients compared to healthy, age-matched controls (Chen et al., 2022; Unger et al., 2016) and inversely correlate with several parameters of disease severity (Aho et al., 2021; Chen et al., 2022). Additional studies have reported a reduction of SCFA-producing bacteria in the PD microbiome (Cirstea et al., 2020; Keshavarzian et al., 2015; Wallen et al., 2021).

One potential target of gut-brain signaling in PD are microglia, resident innate immune cells in the brain parenchyma. Microglia are a highly dynamic population of cells

adopting a spectrum of transcriptomic and morphological phenotypes depending on the condition of their surrounding environment (Masuda et al., 2020). During early development, microglia shape neural circuity through regulation of neurogenesis, synaptic pruning, and myelination (Anderson and Vetter, 2019). In response to injury or disease, microglia upregulate either pro- or anti-inflammatory pathways to clear infections and promote neuronal health. In PD and other neurodegenerative conditions, microglial cellular repair responses are thought to become dysregulated, ultimately resulting in heightened reactivity and chronic inflammation that drives neurodegeneration (Troncoso-Escudero et al., 2018).

Microglia not only sense signals from within the brain but also receive input from the periphery, including the gut microbiome (Abdel-Haq et al., 2019). The absence of signaling between the gut and the brain during fetal development perturbs the development of microglia in mice. Offspring of germ-free (GF) mice show differences in microglial gene expression and chromatin accessibility compared to specific-pathogen-free (SPF) counterparts (Thion et al., 2018). Postnatal signaling along the gut-brain axis is also essential for microglial maturation (Erny et al., 2015; Thion et al., 2018). Microglia from adult GF mice present an immature gene expression profile and fail to adequately respond to immunostimulants. However, feeding GF mice a mixture of acetate, propionate, and butyrate is sufficient to rescue microglial maturation (Erny et al., 2015). In addition, SCFAs exhibit anti-inflammatory properties in a variety of disease models (Cait et al., 2018; Furusawa et al., 2013; H. J. Kim et al., 2007; Vieira et al., 2012). Reduced levels of SCFAs in human PD, coupled with increased

neuroinflammation, suggest that altered production of these metabolites in the gut may link microbiome changes to motor symptoms.

Here, we explored the interplay between diet and microglial physiology in the Thy1-αSyn overexpressing (ASO) mouse model of PD, which recapitulates many of the hallmark symptoms of PD including motor deficits, GI abnormalities, olfactory dysfunction, and neuroinflammation (Chesselet et al., 2012). We demonstrate that a prebiotic diet induces broad changes to the gut microbiome composition and increases SCFA levels in the gut but not the brain. Prebiotic intervention in ASO mice attenuates motor deficits and reduces αSyn aggregates in the substantia nigra in a microglia-dependent manner. Increased SCFA production is associated with changes in the morphology and gene expression patterns of microglia in brain regions involved in PD pathology, in a manner suggestive of decreased microglial reactivity to αSyn. Overall, this study reveals that enhanced metabolism of dietary fiber by the gut microbiome alters the physiology of cells in the central nervous system (CNS) and ultimately improves pathological and behavioral outcomes in a mouse model of PD.

Results

Prebiotic diet attenuates motor symptoms and reduces αSyn aggregation in the brain

To test whether prebiotics could attenuate disease pathology in ASO mice, we generated three custom high-fiber diets **(Supplement Table 1)**, each containing 20% of a prebiotic mixture of two or three dietary fibers designed to promote distinct groups of bacterial taxa in the large intestine **(Supplement Figure 1A)** and boost SCFA production (**Supplement Figure 1B-E)** based on an *in vitro* fecal fermentation study. The prebiotic diets **(Supplement Figure 1F)** were compared to a cellulose-free control diet.

We fed each of the three prebiotic diets (prebiotic #1, #2, #3) to male ASO mice from 5-22 weeks of age. To assess whether long-term prebiotic intervention ameliorated motor deficits, mice were subjected to a battery of motor tests at 22 weeks to evaluate fine motor control, grip strength, locomotion and coordination **(Figure 1A-D, Supplement Figure 2A-G)**. From this screening experiment we identified a single prebiotic (prebiotic #1, referred to hereafter as "prebiotic") that improved disease symptoms in ASO mice. Remarkably, administration of the prebiotic diet to ASO mice improved performance in several motor behavioral tests, including the pole descent and beam traversal tests (time to cross, steps to cross, errors per step) compared to mice fed a control diet **(Figure 1A-D).** Performance in other paradigms including adhesive removal, wire hang, and hindlimb score was unchanged **(Supplement Figure 3A-C)**. These findings suggest that gut-targeting therapeutics have the potential to attenuate key pathological features of PD.

However, the different neural circuitries that modulate specific motor tasks may be differentially sensitive to microbiome-targeted dietary interventions.

Levels of all tested SCFAs were higher in fecal samples from prebiotic-fed mice than from control-fed mice **(Figure 1E)**. Interestingly, concentrations of propionate, butyrate, and isobutyrate were not significantly different between wild type (WT) and ASO mice fed a control diet, while levels of acetate were significantly increased in control-ASO mice **(Figure 1E)**. To further characterize the effects of the prebiotic diet on ASO mice, we measured food intake throughout the duration of the experiment and body weight at 22 weeks of age. ASO mice weighed significantly less than their WT counterparts and exhibited reduced food intake **(Supplement Figure 3D-E).** Interestingly, while prebiotic-ASO mice ate significantly more than control-ASO mice, no difference in body weight was detected between the groups at 22 weeks **(Supplement Figure 3D)**.

Aggregation of αSyn is a hallmark of PD pathology. Since previous work found that absence of the gut microbiome decreases αSyn aggregation in ASO mice (Sampson et al., 2016), we tested whether a prebiotic diet would exert similar effects. Notably, we found a significant reduction in αSyn aggregation in the substantia nigra (SN) of prebiotic-fed ASO mice compared to ASO mice on control chow **(Figure 1F).** In contrast, prebiotic intervention had no effect on αSyn aggregation in the striatum (STR) **(Figure 1G).** We speculate that this difference may be attributable to regional differences in microglia density, gene expression, and clearance activity, with the SN having a relatively higher density of microglia (Grabert et al., 2016; Y.-L. Tan, Yuan, and Tian, 2020). Taken

together, these results suggest that early intervention with a prebiotic diet can affect key features of PD pathology in ASO mice, including motor deficits and αSyn aggregation.

Prebiotics alter gut microbiome composition

The gut microbiome composition is strongly influenced by diet in mice and humans (Turnbaugh et al., 2009; Wu et al., 2011). To investigate whether prebiotic intervention reshapes gut microbial profiles, we performed shotgun metagenomics. Alpha diversity analysis revealed significant reduction in observed species count, Shannon's diversity, and Simpson's evenness in prebiotic-fed groups, as well as an increase in Gini's dominance **(Figure 2A-D)**. This is consistent with a previous report of reduced microbiome diversity in high-fiber fed mice (Luo et al., 2017). Principal coordinate analysis (PCoA) of species abundance showed that samples clustered more closely by diet treatment than mouse genotype (**Figure 2E)** and PERMANOVA analysis revealed that prebiotic treatment explained 6-fold more variance than genotype, with R^2 values of 0.334 and 0.053 for each, respectively **(Figure 2F).** Thus, the prebiotic diet is a powerful driver of gut microbial community structure in both WT and ASO mice**.**

A closer look at microbiome composition revealed broad changes at the phylum and genus levels **(Figure 2G, I).** We observed an increase in Bacteroidetes and a decrease in Firmicutes in prebiotic diet-fed mice, resulting in a lower Firmicutes/Bacteroidetes (F/B) ratio **(Figure 2H)**. A high F/B ratio has been shown to correlate with metabolic disorders including obesity and high fat intake in several studies (Ley et al., 2006; Magne et al.,

2020), although this trend has not been observed in other human studies (Duncan et al., 2008; Walters, Xu, and Knight, 2014). Bacteroidetes are also reduced in PD patients compared to age-matched controls (Unger et al., 2016). Additionally, we observed a decrease in Proteobacteria, a phylum often increased in dysbiosis and inflammation and elevated in PD patient fecal samples **(Figure 2H)** (Keshavarzian et al., 2015; Shin, Whon, and Bae 2015). Gut-brain module analysis showed variation in metabolic pathways including SCFA degradation/synthesis in response to diet and genotype **(Figure 2J)**. Overall, feeding of a prebiotic diet appears to qualitatively restructure the ASO microbiome toward an anti-inflammatory and potentially protective profile.

Prebiotic diet alters microglia morphology and reactivity status in ASO mice

In ASO mice, microglia reactivity in response to α-synuclein overexpression appears at 4-5 weeks of age in the STR and at 20-24 weeks of age in the SN (Watson et al., 2012). SCFAs have been shown to influence the physiology of microglia in several contexts (Colombo et al., 2021; Erny et al., 2015; Sadler et al., 2020; Erny et al., 2021; Sampson et al., 2016). To explore whether prebiotics alter microglia morphology, we performed immunofluorescence imaging of IBA1, a pan-microglial marker. The morphology of microglia can indicate their reactivity state, with homeostatic microglia exhibiting a ramified shape with a smaller cell body and increased dendritic processes and microglia responding to external stimuli adopting an amoeboid form with a larger cell body and retracted processes (Menassa and Gomez-Nicola, 2018). We observed that microglia in the SN and STR of prebiotic-ASO mice had significantly smaller cell bodies than in control-ASO mice (**Figure 3A-B)**. 3D analysis of key morphological features revealed

that microglia in the SN and STR of prebiotic-ASO mice exhibited increased dendrite length, number of segments, number of branch points, and number of terminal points compared to microglia from control-ASO mice **(Figure 3C-F).** Taken together, these findings indicate that long-term prebiotic intervention dampens microglial reactivity in regions of the brain implicated in PD.

Prebiotic diet alters microglia gene expression

Single cell RNA sequencing (scRNA-seq) has emerged as a powerful tool to interrogate microglial biology in mouse models of neurodegeneration (Keren-Shaul et al., 2017; W. Liu et al., 2020). We therefore decided to do scRNA-seq to transcriptionally profile microglia in PD-relevant brain regions of control and prebiotic-fed WT and ASO mice.

Based on global gene expression, Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) analysis yielded nine distinct microglia clusters in the SN and eight clusters in the STR **(Figure 4A, F)**. In the SN, we detected differences in cluster distribution between experimental groups, with the strongest differences in clusters 0 and 2 **(Figure 4A-B)**. Interestingly, the percentage of microglia in cluster 0 was higher in control-ASO than control-WT mice (27.1% vs. 18.9%), and prebiotic treatment reduced the percentage of microglia belonging to cluster 0 in ASO mice compared to control diets (18.3%) **(Figure 4B)**. Gene enrichment analysis of the top 50 genes associated with cluster 0 revealed pathways related to immune system processes, cellular response to tumor necrosis factor, cellular response to lipopolysaccharide, and response to stress. Cluster 0 contained several prominent immune markers including *Tnf*, *Nfkbia*,

Ccl2, *Ccl3*, and *Ccl4*, suggesting that a prebiotic diet may suppress or prevent pro-inflammatory processes in response to αSyn overexpression. Notably, levels of TNF and Ccl2 are elevated in the serum of PD patients (Brodacki et al., 2008; Reale et al., 2009). We observed the opposite trend in cluster 2. The percentage of microglia belonging to cluster 2 was reduced in control-ASO mice but increased in prebiotic-ASO mice **(Figure 4B)**. Among the most highly expressed genes in cluster 2 were the homeostatic microglial markers *P2ry12* and *Cst3*, as well as the anti-inflammatory transcription factors *Klf2* and *Klf4* (Das et al., 2006; Li et al., 2018).

Within the STR, we detected eight clusters of microglia, with notable shifts in clusters 1 and 3 **(Figure 4F-G)**. The top 10 associative genes in cluster 3 included several mitochondrial genes*: mt-Atp6, mt-Cytb, mt-Co2, mt-Co3, mt-Nd4, mt-Nd1*, and *mt-Nd2*. Additionally, we detected a 13.4% increase in microglia in cluster 1 in control-ASO mice, with prebiotic diet restoring the percentage of cluster 1 back to control-WT levels **(Figure 4G)**. The significantly enriched pathways within cluster 1 included those positively regulating cell death and immune system development, and negatively regulating cellular processes, suggesting increased immune signaling and dysregulation of homeostatic signaling.

Next, we sought to investigate differences in microglial gene expression between control-WT and control-ASO mice. Differential gene expression analysis of all cells revealed 313 differentially expressed genes (DEGs) (↑163, ↓150, FDR<0.05) in the SN and 997 DEGs (↑511, ↓486) in the STR. In the SN, microglia harvested from control-ASO mice

displayed increased expression of MHC class I components (*H2-K1, H2-D1*), several chemokines (*Ccl2, Ccl3, Ccl4, Ccl9*) and chemokine receptors (*Ccr1, Ccr5*), and pro-inflammatory markers (*Nfkbid, CD74)* **(Figure 4C, Supplement Table 2)**. Gene enrichment analysis of all upregulated DEGs in control-ASO mice showed enrichment in pathways related to cellular responses to cytokine stimulus and interferon-gamma, immune system processes, and response to stress **(Figure 4D)**. Interestingly, several genes that were downregulated in control-ASO mice compared to control-WT were related to anti-inflammatory signaling (*Klf2, Klf4*) and microglial homeostasis (*P2ry12, Slc2a5*) **(Figure 4C, Supplement Table 2)**. We observed similar trends in the STR, with control-ASO microglia upregulating pro-inflammatory modulators (*Tnf*, *Nfkbiz, Trim8, Irgm1*) and antigen processing and presentation genes (*H2-Q7*, *H2-K1*, *H2-D1*, *H2-T23*) and downregulating genes related to homeostatic cellular processes **(Figure 4H-I, Supplement Table 4)**. Notably, the anti-inflammatory cytokine transforming growth factor beta 2 (*Tgfb2*) was ~45-fold downregulated in control-ASO **(Supplement Table 4)**. This data suggests microglia from control-ASO mice upregulate pro-inflammatory immune processes and downregulate pathways related to homeostasis and cellular maintenance in response to αSyn pathology.

To assay effects of long-term prebiotic intervention on microglial gene expression in ASO mice, we compared prebiotic-ASO microglia to control-ASO and found 473 DEGs (↑317, ↓156) in the SN and 1,474 DEGs (↑608, ↓866) in the STR **(Figure 4C, H, Supplement Tables 3, 5).** Gene enrichment analysis of the 156 genes downregulated in prebiotic-ASO microglia in the SN revealed reduction in IL-1β production pathways, as

well as dampened innate immune response and defense response pathways (**Figure 4E)**. Among the genes downregulated in prebiotic-ASO relative to control-ASO microglia were several mediators of the pro-inflammatory immune response (*Mif, Masp1, Trim12a, Bs2, B2m*), antigen presentation and processing (*H2-Q7*), and chemokines/receptors (*Ccl9, Ccr1, Ccr5*) **(Figure 4C, Supplement Table 3)**. We observed a similar trend in the STR, with prebiotic-ASO showing downregulation of pathways related to innate immunity, response to stress, and defense response **(Figure 4H, J, Supplement Table 5)**. Interestingly, several of the pro-inflammatory markers upregulated in control-ASO and downregulated in prebiotic-ASO microglia were expressed by a small subset of microglia, suggesting that a subpopulation of cells alters its transcriptomic profile in response to αSyn expression, similar to what has been observed in microglia from aged mice and the 5XFAD mouse model of Alzheimer's disease (AD) (Hammond et al., 2019; Keren-Shaul et al., 2017). Further DEG analysis revealed increased expression of several disease-associated microglia (DAM) markers in the SN and STR in prebiotic-ASO mice (**Supplement Tables 3, 5)**. In the STR we observed an increase in Trem2 in microglia from prebiotic-ASO mice, suggesting prebiotics may induce a neuroprotective DAM phenotype at 22 weeks of age (Gratuze, Leyns, and Holtzman, 2018; Keren-Shaul et al., 2017; Onuska, 2020). Taken together, our analysis suggests prebiotic intervention in ASO mice dampens proinflammatory and neurotoxic signaling pathways and potentially upregulates a neuroprotective phenotype in microglia.

Potential effects of SCFAs are likely indirect and not via epigenetic regulation

Next, we sought to explore whether changes in microglial morphology or gene expression were prompted by changes in SCFA levels or signaling in the brain. SCFAs can cross the BBB and have been detected in the cerebrospinal fluid and brains of mice and humans (Bachmann, Colombo, and Berüter, 1979; Frost et al., 2014). However, we detected no differences in SCFA levels between control and prebiotic animal groups in either the SN or STR **(Supplement Figure 4A-B)**. We therefore investigated SCFA signaling in the brain. SCFAs signal through activation of GPCR receptors (GPCR43 or FFAR2, and GPCR41 or FFAR3) and/or inhibition of histone deacetylases (HDACs), altering the epigenetic landscape of target cells (Silva, Bernardi, and Frozza, 2020; Vinolo et al., 2011). Accordingly, we measured expression of FFAR2 and FFAR3 in the cerebellum, midbrain, striatum, motor cortex, and small intestine by qPCR. All four brain regions exhibited very low or no expression of FFAR2 and FFAR3 relative to the small intestine **(Supplement Figure 5A, B).** This was consistent with our scRNA-seq data, which showed an absence of FFAR2/3 expression in microglia in the SN and STR.

To explore whether our prebiotic diet was inducing epigenetic changes, we performed bulk ATAC-seq on purified microglia from the SN and STR of control and prebiotic-fed mice but saw no significant difference in chromatin accessibility between experimental groups **(Supplement Figure 5C, D)**. However, from this bulk measurement, we cannot rule out changes in open chromatin or histone modifications in specific subset(s) of microglia. We also measured the expression levels of several HDAC isoforms (*HDAC 1,2,6,7, and 9*) in the striatum and found no difference in expression between control and

prebiotic groups of both genotypes **(Supplement Figure 5E-I)**. Collectively, these findings suggest that elevated levels of SCFAs resulting from prebiotic intervention may influence microglial morphology and gene expression through indirect mechanisms, although additional work is needed to validate this hypothesis.

Depletion of microglia blocks beneficial effects of prebiotics on motor performance and α-synuclein aggregation

Given the observed differences in microglial morphology and gene expression in prebiotic-ASO mice, we next sought to determine whether prebiotic-mediated attenuation of motor deficits is dependent on microglial signaling. Microglia are dependent on colony stimulating factor 1 receptor (CSF1R) signaling for development, maintenance, and proliferation (Elmore et al., 2014). PLX5622 is a brain-penetrant inhibitor of CSF1R and was previously reported to deplete microglia with no effect on behavior or cognition (Elmore et al., 2014). We administered PLX5622 in the diet of mice from 5-22 weeks of age, and quantified the number of IBA1+ microglia in various brain regions. Notably, the efficiency of microglial depletion varied depending on brain region, with regions containing low numbers of microglia such as the cerebellum exhibiting higher depletion (~80%) than areas with high microglial density such as the SN (~65%) and STR (~75%) **(Figure 5A-C).** We did not observe differences in depletion efficiency between WT and ASO mice or between control and prebiotic-fed mice **(Supplement Figure 6A-B)**.

Following PLX5622 treatment, we assayed motor behavior at 22 weeks of age. PLX5622 treatment had no impact on motor performance in tests where prebiotic treatment had no

effect **(Supplement Figure 6C-F).** Remarkably however, even incomplete microglia depletion eliminated prebiotic-induced improvements in the pole descent and beam traversal tests (time to cross, errors per step) **(Figure 5D-F)**, suggesting that microglia mediate the ability of prebiotics to ameliorate motor deficits. PLX5622 treatment did not alter body weight in control or prebiotic-fed mice **(Supplement Figure 6G)**. We also measured αSyn aggregation in the SN and STR of 22-week-old mice. In control-fed mice, depletion of microglia had no impact on levels of αSyn aggregation in the SN or STR **(Figure 5G-H).** However, in prebiotic-fed ASO mice, depletion of microglia significantly increased levels of aggregated αSyn in the SN, while levels in the STR remained unchanged **(Figure 5G-H)**. These data reveal that partial ablation of microglia and diminished CSF1R signaling eliminate the protective effects of the prebiotic diet in ASO mice.

Certain peripheral immune cell types are also reliant on CSF1R signaling for their proliferation and survival. While previous studies have characterized the effect of PLX5622 on macrophages in the spleen and bone marrow (Lei et al., 2020), knowledge of the effect of this drug on immune cell populations in the gastrointestinal tract of mice is limited. Surprisingly, most of the immune cell populations we characterized at 22 weeks of age were unaffected by PLX5622 treatment. In the large intestine, PLX5622 treatment caused a reduction in $CD45^{+}$ $CSF1R^{lo}$ lymphocytes, but had no impact on $CD45^{+}$ $CSF1R^{hi}$ cells, pan T cells or B cells **(Supplement Figure 7A-E).** In the small intestine, levels of these cell types were unchanged in response to PLX5622 **(Supplement Figure 7F-J)**. In the spleen, while $CSF1R^{lo}$ lymphocytes were reduced in

Prebiotic + PLX5622 mice, levels of CSF1R^{hi} macrophages were significantly elevated in Control + PLX5622 and Prebiotic + PLX5622 mice, suggesting a potential compensatory mechanism in this organ **(Supplement Figure 7K-O)**. These findings point to a relatively high specificity of CSF1R-targeted depletion for microglia, further implicating microglia as a key mediator of the beneficial effects of prebiotic treatment in ASO mice.

Discussion

Herein, we describe how a prebiotic diet that increases SCFA levels in αSyn overexpressing mice results in improved motor performance and reduced microglial reactivity and αSyn pathology. The mechanism by which SCFAs influence microglial physiology and alter behavior remains unclear. SCFA levels in the brain tissue of prebiotic-fed mice were unchanged, suggesting that elevated SCFAs in the GI tract or circulation may influence other organ systems, initiating a cascade of events that ultimately impacts microglia. SCFAs are known to have immune modulatory effects in the gut (Parada Venegas et al., 2019), among other functions, and we speculate that altering peripheral immunity may indirectly affect microglia reactivity states and gene expression. Moreover, we found that SCFAs do not appear to signal through known GPCRs in the brain or via epigenetic remodeling of microglia-derived chromatin, further reinforcing the notion that SCFAs do not directly interact with microglia, as previously suggested (Erny et al., 2015). We note that it is also possible that another class of microbial metabolites may be contributing to prebiotic-induced changes in microglial physiology.

Studies of SCFAs in preclinical models paint a complex picture, with SCFAs exhibiting varying properties in germ-free (GF) vs. SPF settings. Oral administration of SCFAs to GF mice induces microglial reactivity in wild-type mice (Erny et al., 2015), a mouse model of AD (Colombo et al., 2021), and ASO mice, where feeding the metabolites in the absence of gut bacteria exacerbates motor deficits and neuroinflammation (Sampson et al., 2016). In contrast, two independent studies found that sodium butyrate treatment alleviates motor deficits and reduces microglial reactivity in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice with a laboratory microbiota (Hou et al., 2021; J. Liu et al., 2017). Our studies herein corroborate that SCFAs are associated with anti-inflammatory profiles in microglia from SPF mice, and underscore the need for caution in future studies to consider context (GF vs. SPF), concentration, and duration of SCFA interventions in mice.

Microglia are gaining increasing attention for their role in neurodegenerative and related disorders. Depletion of microglia using CSF1R inhibitors confers deleterious effects in certain mouse models of PD **(**MPTP, human α-syn AAV) (George et al., 2019; X. Yang et al., 2018), LPS-induced sickness behavior (Vichaya et al., 2020), and prion disease (Carroll et al., 2018). In contrast, microglia depletion improves disease outcome in experimental autoimmune encephalomyelitis (EAE), a preclinical models of multiple sclerosis (Nissen et al., 2018), and in 3xTg and 5xFAD AD mouse models (Casali et al., 2020; E. E. Spangenberg et al., 2016; E. Spangenberg et al., 2019). In this study, we found that depletion of microglia neither exacerbates nor improves motor performance in

naïve (control diet) mice, suggesting that microglia do not influence behavior in ASO mice, at least in early stages of disease progression. In contrast, the protective effects of a prebiotic diet do require microglia since their depletion eliminated improvements in motor behavior and αSyn pathology in the brain.

We extended these findings with scRNA sequencing, uncovering functional effects including restoration of pathways known to be dysregulated in PD including inflammation and homeostatic cellular functions. Moreover, we found that prebiotic intervention significantly increases CSF1 expression in ASO microglia in both the SN and STR, potentially implicating CSF1 signaling pathways in mediating the protective effects of prebiotics. Further insights into how prebiotic diets modulate microglia biology and how these events translate into amelioration of motor symptoms and brain pathology await future research. Microglia have been shown to present a distinct transcriptomic profile and respond to various environmental factors, including the microbiome, in a sex-specific manner (Thion et al., 2018; Villa et al., 2018). While this study probed the effects of prebiotics on microglia in male mice, additional insight may come from similar investigation of female mice.

In the last decade, identification of a link between the microbiome and microglial physiology has opened possibilities for the potential treatment of neurological conditions such as PD. Gut-microbiome-based therapies encompass prebiotics, probiotics, and fecal microbiota transplant (FMT). Prebiotics present a particularly promising approach, as diet is a significant contributor to microbiome composition, and epidemiological evidence has

revealed a link between diet and risk of developing PD (Boulos et al., 2019). While increased intake of fruits, vegetables, and adherence to a Mediterranean diet are associated with a lower risk of PD, individuals consuming a low-fiber, highly-processed Western diet exhibit an increased risk of PD diagnosis (Alcalay et al., 2012; Gao et al., 2007; Molsberry et al., 2020). Several ongoing clinical trials are exploring the beneficial effects of probiotics and prebiotics on PD-related outcomes. Gut-targeted therapies offer several advantages compared to traditional therapeutic approaches for brain disorders. Conventional pharmacological treatments rely on chemicals which may lose efficacy over time, often fail to treat underlying pathophysiology, and may result in undesirable side effects for the patient. Diet- or microbiome-based therapies, in contrast, may offer the added advantage of improving overall health. Another notable challenge for CNS-targeting drugs is delivery, requiring drugs that can efficiently cross the blood-brain barrier. Harnessing safe and tolerable treatment options based on diet may therefore help accelerate novel therapeutics for PD.

Figures and Legends

Figure 1: Prebiotic diet attenuates motor symptoms, increases fecal SCFA levels, and reduces αSyn aggregation in the brain

Figure 1A-D: Motor behavior metrics at 22 weeks of age in prebiotic- and control-fed WT and ASO mice in the B) Pole descent test C) Beam-time to cross D) Beam-steps to cross and E) Beam-errors per step. Motor test data is derived from two independent experiments (n=16-29/group). Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001

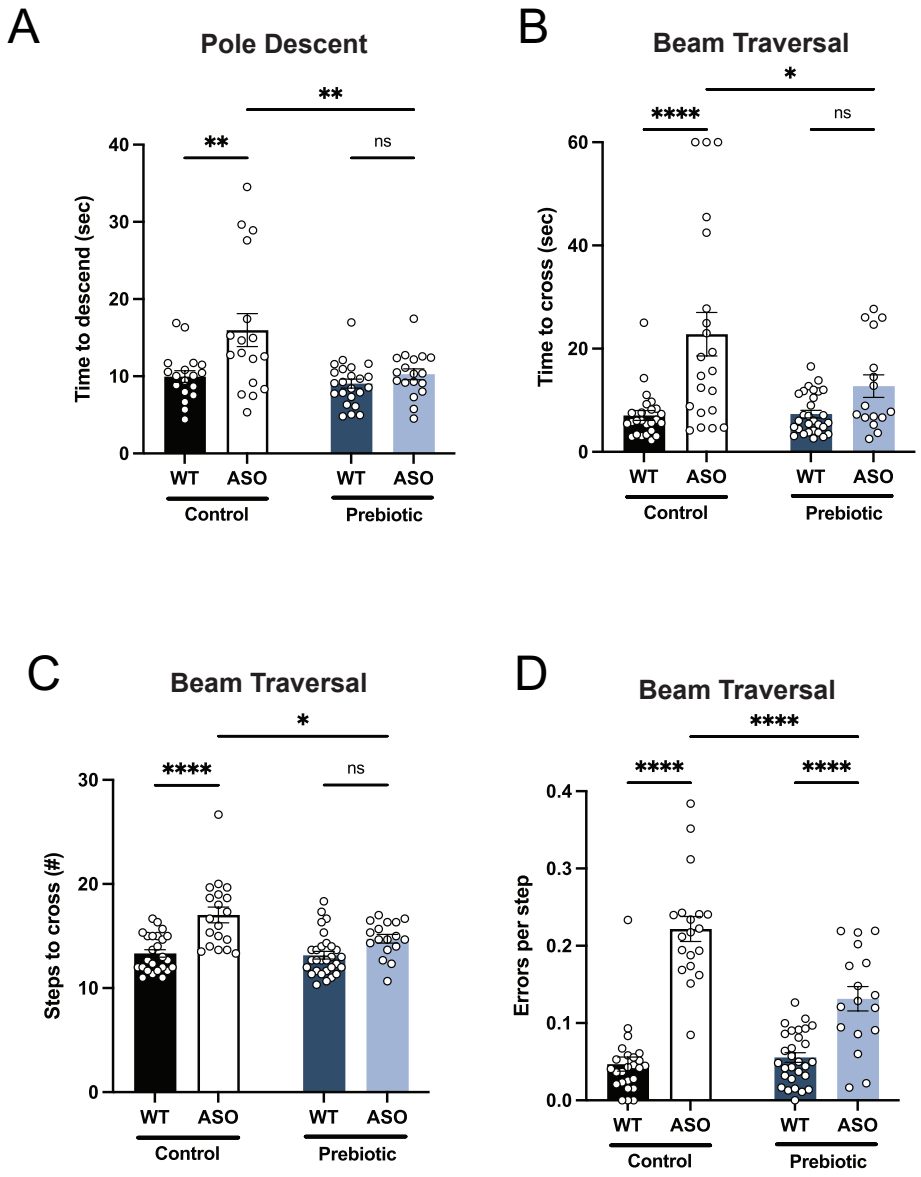


Figure 1: Prebiotic diet attenuates motor symptoms, increases fecal SCFA levels, and reduces αSyn aggregation in the brain

Figure 1E: Concentration (μM) of acetate, propionate, butyrate, and isobutyrate in fecal samples collected from prebiotic-fed WT and ASO mice (n=7-12/group). Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001

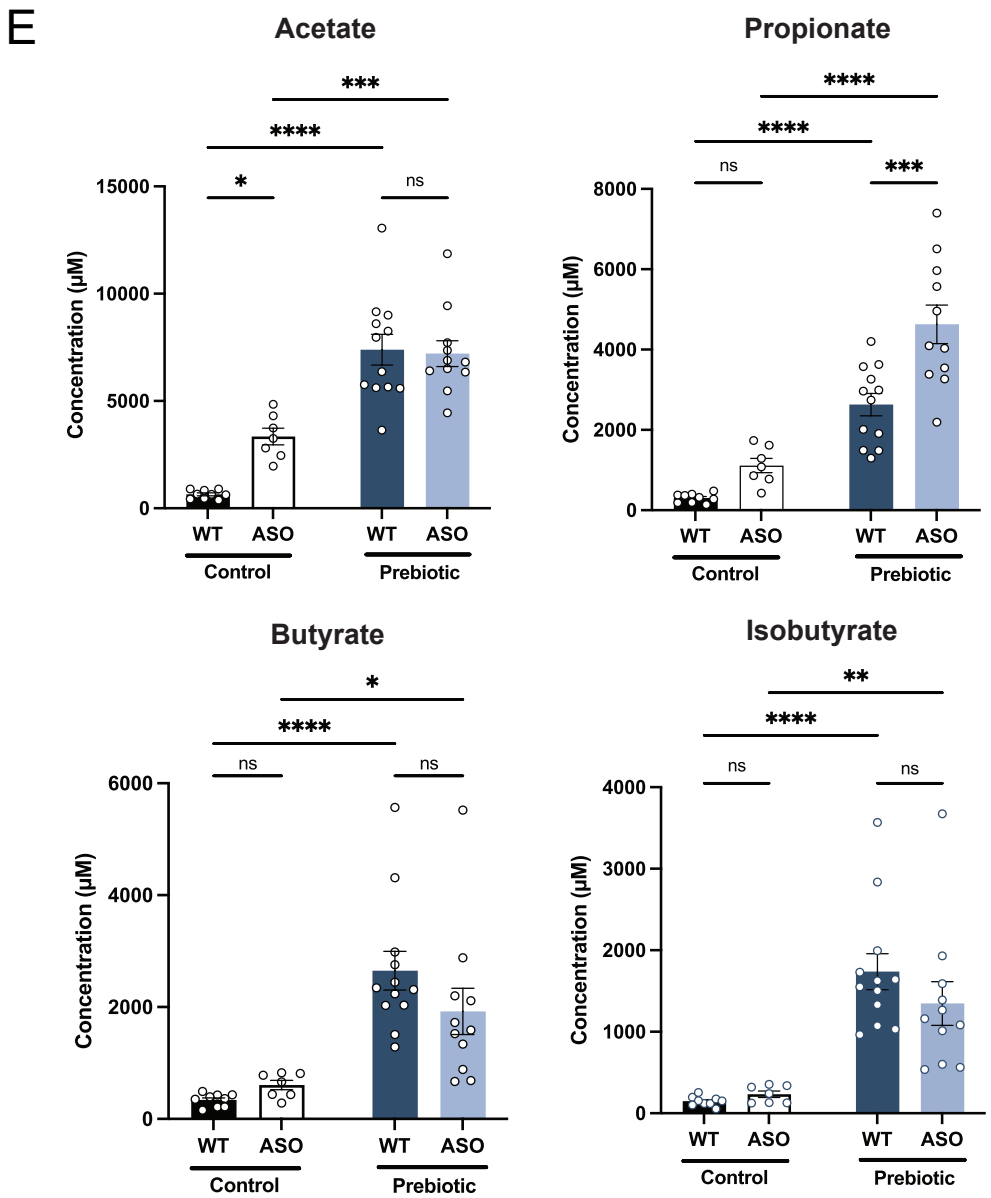


Figure 1: Prebiotic diet attenuates motor symptoms, increases fecal SCFA levels, and reduces αSyn aggregation in the brain

Figure 1F-G: Aggregated α-synuclein levels in the F) Substantia Nigra (n=8-10/group) but not in the G) Striatum (n=9-11/group) measured by dot blot. Each point represents data from one individual mouse. Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001

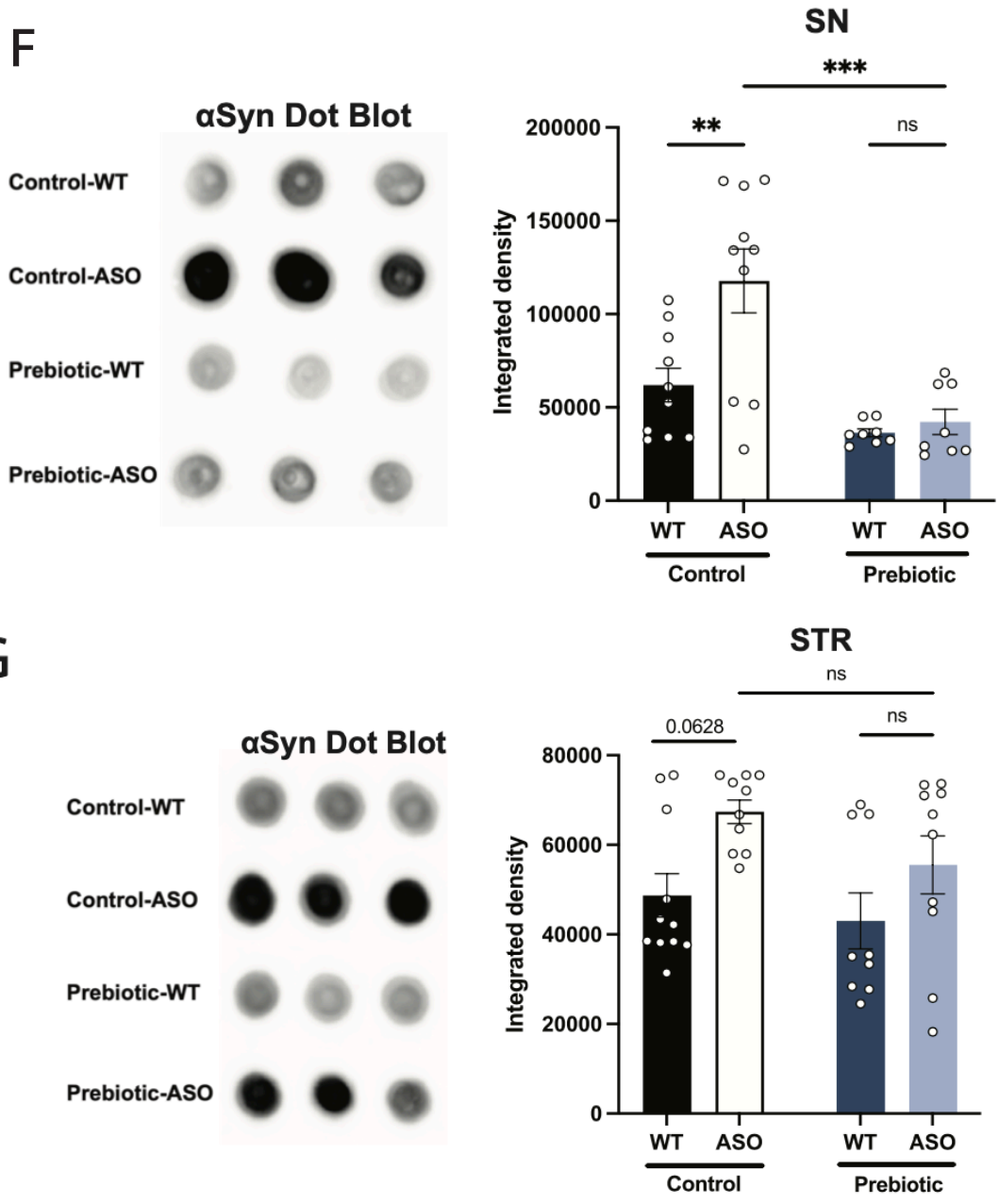


Figure 2: Prebiotic diet alters gut microbiome composition

Figure 2A-D: Diversity metrics from metagenomic analysis of all treatment groups at 22 weeks of age including A) Observed species count B) Shannon's diversity C) Simpson's evenness, and D) Gini's dominance. (n=12-25/group)

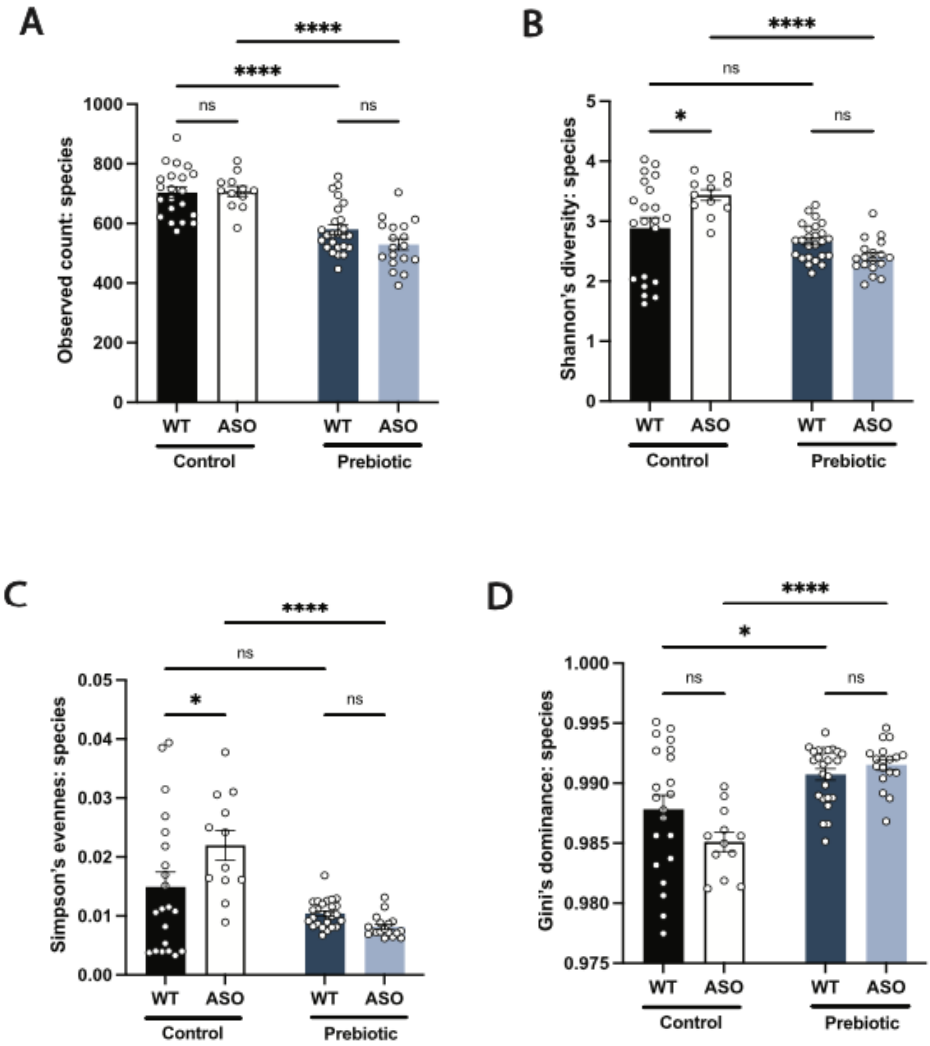


Figure 2: Prebiotic diet alters gut microbiome composition

Figure 2E-F: E) PCoA plot of Bray-Curtis dissimilarity F) PERMANOVA analysis summary of Bray-Curtis dissimilarity. (n=12-25/group).

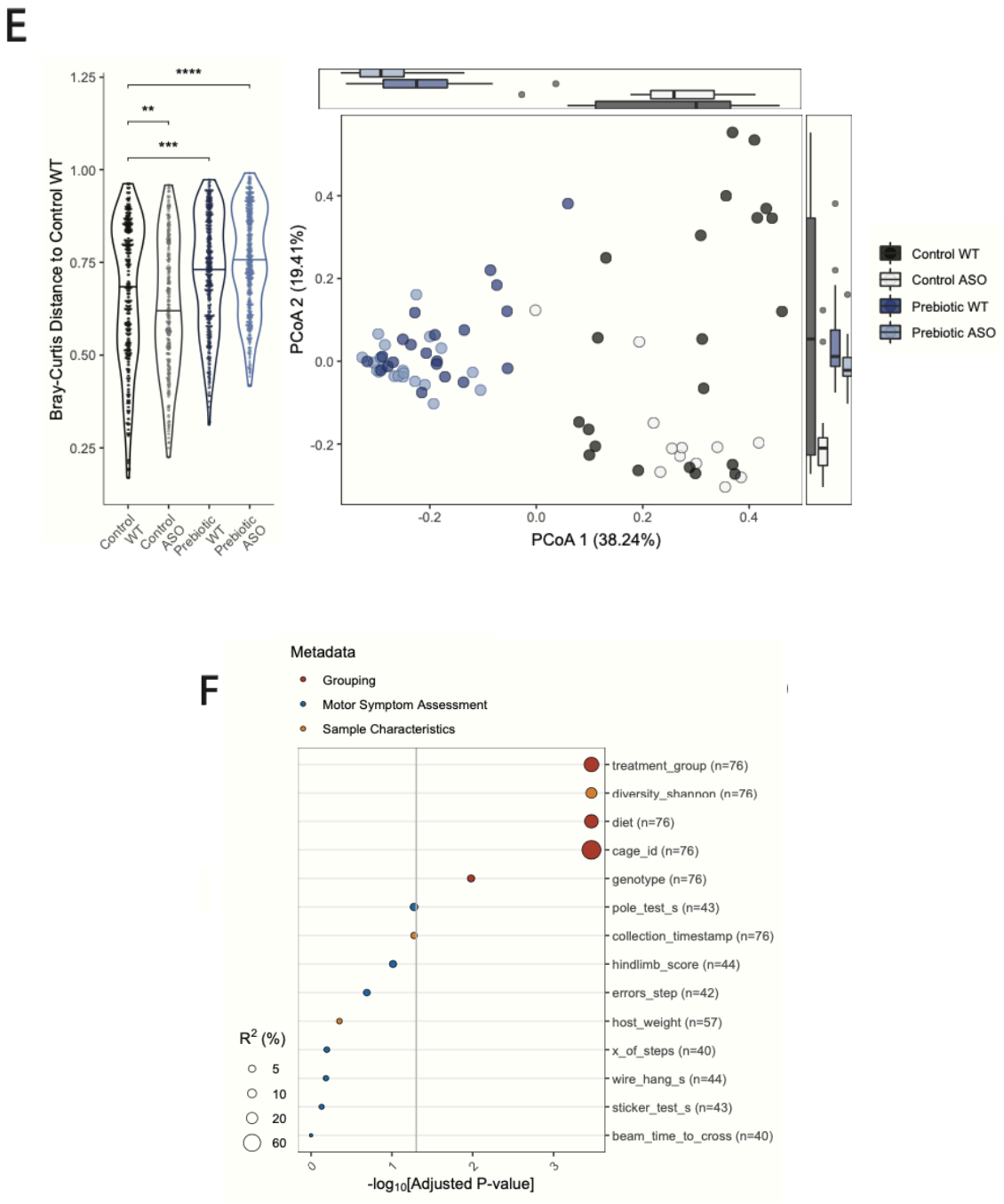
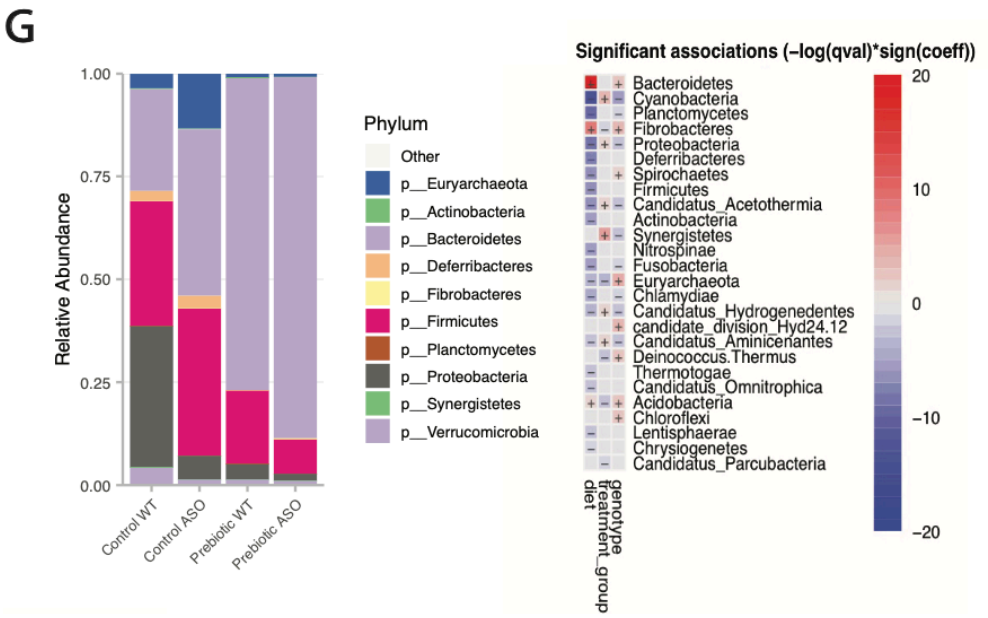


Figure 2: Prebiotic diet alters gut microbiome composition

Figure 2G-H: G) left) Relative abundance of phyla among treatment groups right) Heat map demonstrating differentially abundant phyla. Diet values displayed relative to Control diet and genotype values displayed relative to WT mice. H) Relative abundance of select phyla in treatment groups. (n=13-25/group).



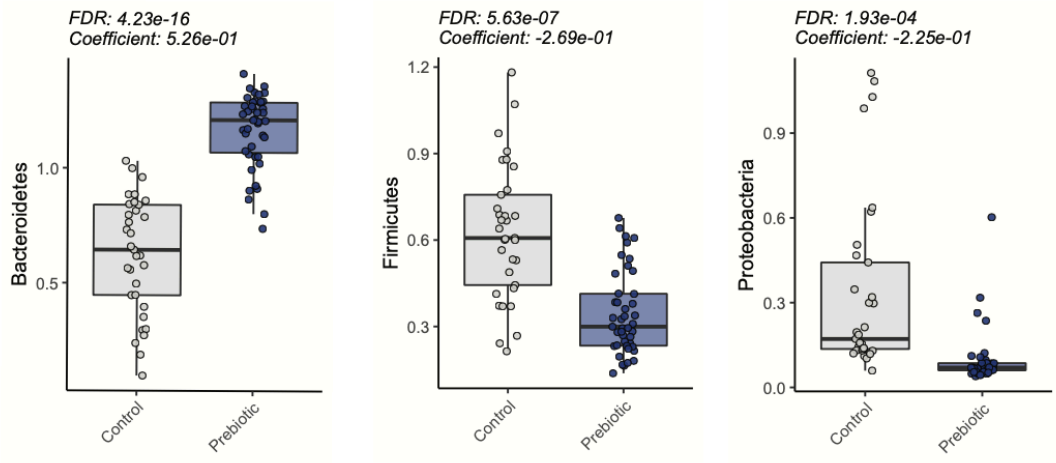


Figure 2: Prebiotic diet alters gut microbiome composition

Figure 2I-J: I) Summary plot of relative abundance of genera. J) Gut Microbiome-Brain Module heatmap with differentially expressed pathways. Diet values displayed relative to Control diet and genotype values displayed relative to WT mice. (n=13-25/group).

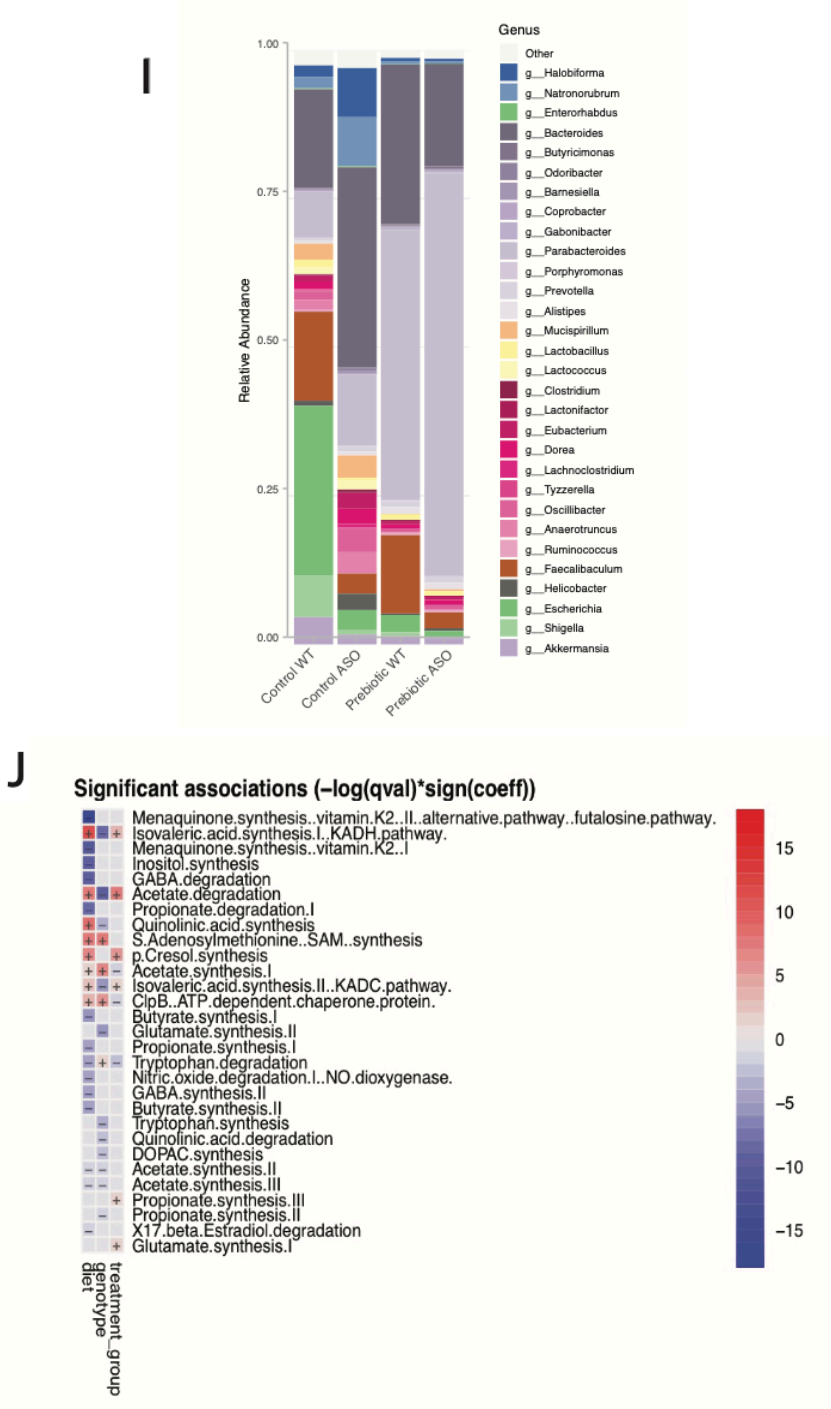


Figure 3: Prebiotic diet alters microglia morphology and reactivity status in ASO mice

Figure 3A-B: Measurement of IBA1+ microglia diameter in A) Substantia nigra (n=5/group) and B) Striatum (n=5/group); Left: quantification of cell diameter. Each point represents one mouse with 26-79 cells measured per mouse. Right: Representative 20x images of IBA1+ staining. Scale bar 50μm. Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001

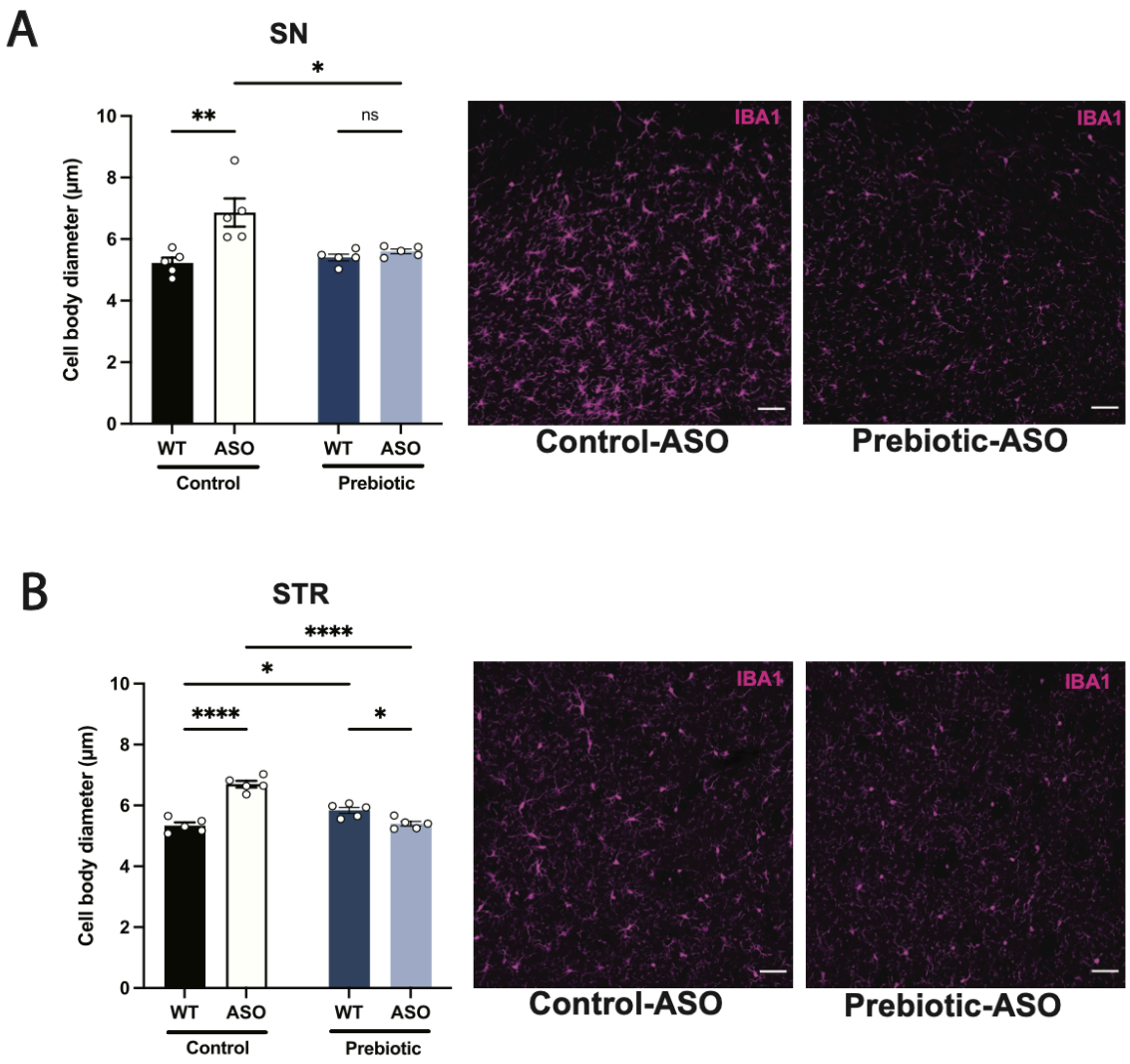
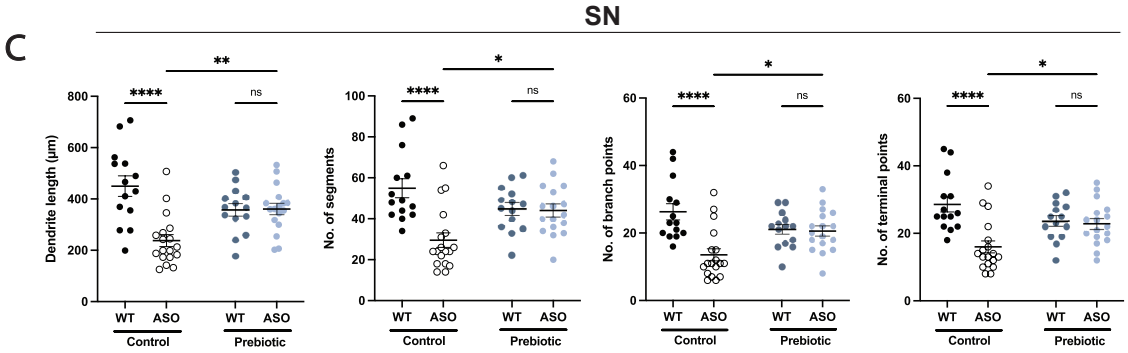


Figure 3: Prebiotic diet alters microglia morphology and reactivity status in ASO mice

Figure 3C-D: 3D reconstruction of microglia using Imaris software in C) Substantia nigra: dendrite length, number of segments, number of branch points, number of terminal points (n=14-18/group) D) representative image of 3D reconstructed cells in the Substantia Nigra; magnification 40x. Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



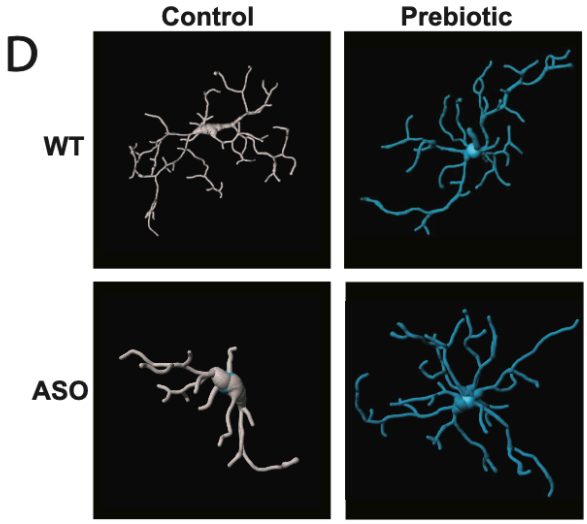
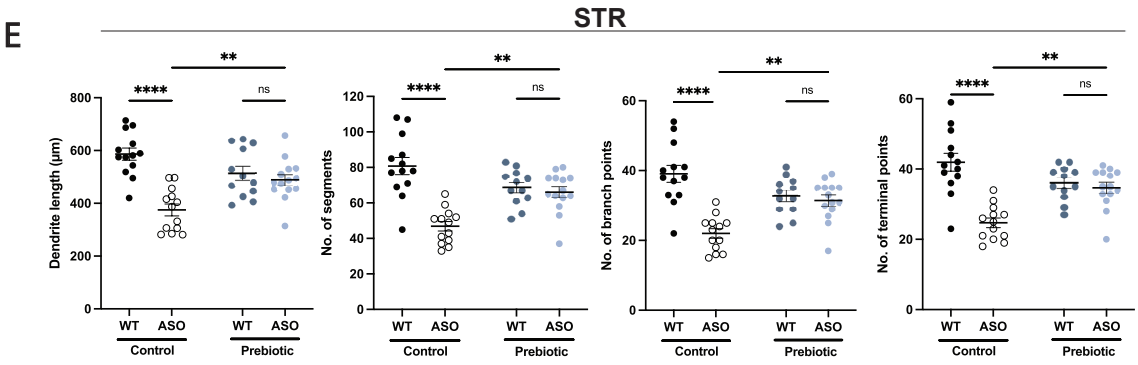


Figure 3: Prebiotic diet alters microglia morphology and reactivity status in ASO mice

Figure 3E-F: 3D reconstruction of microglia in the E) Striatum: dendrite length, number of segments, number of branch points, number of terminal points (n=12-14/group). Each point represents one cell, with 3-5 cells analyzed/mouse. F) Representative image of 3D reconstructed cells in the striatum; magnification 40x. Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



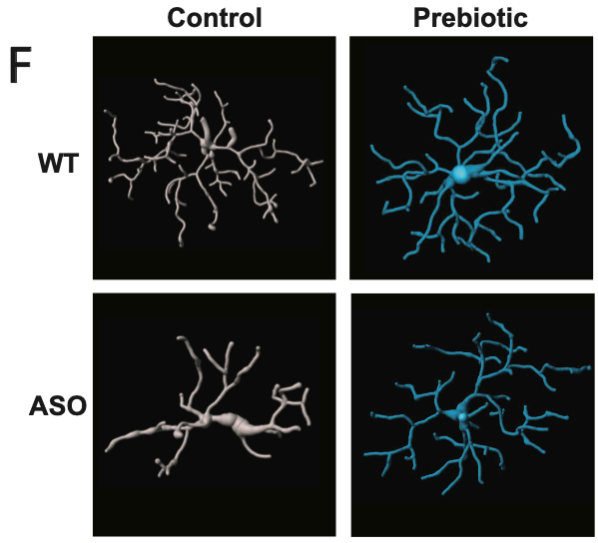


Figure 4: Prebiotic diet alters microglia gene expression

Figure 4A-B: scRNA-seq of the four experimental groups reveals changes in cluster distribution and gene expression between experimental groups in both the Substantia Nigra (SN) and Striatum (STR) A) Left) UMAP plot of all cells sequenced in the in the SN (n=5278 cells). Right) Distribution of cells in individual samples B) Relative distribution of cells within each cluster in the SN.

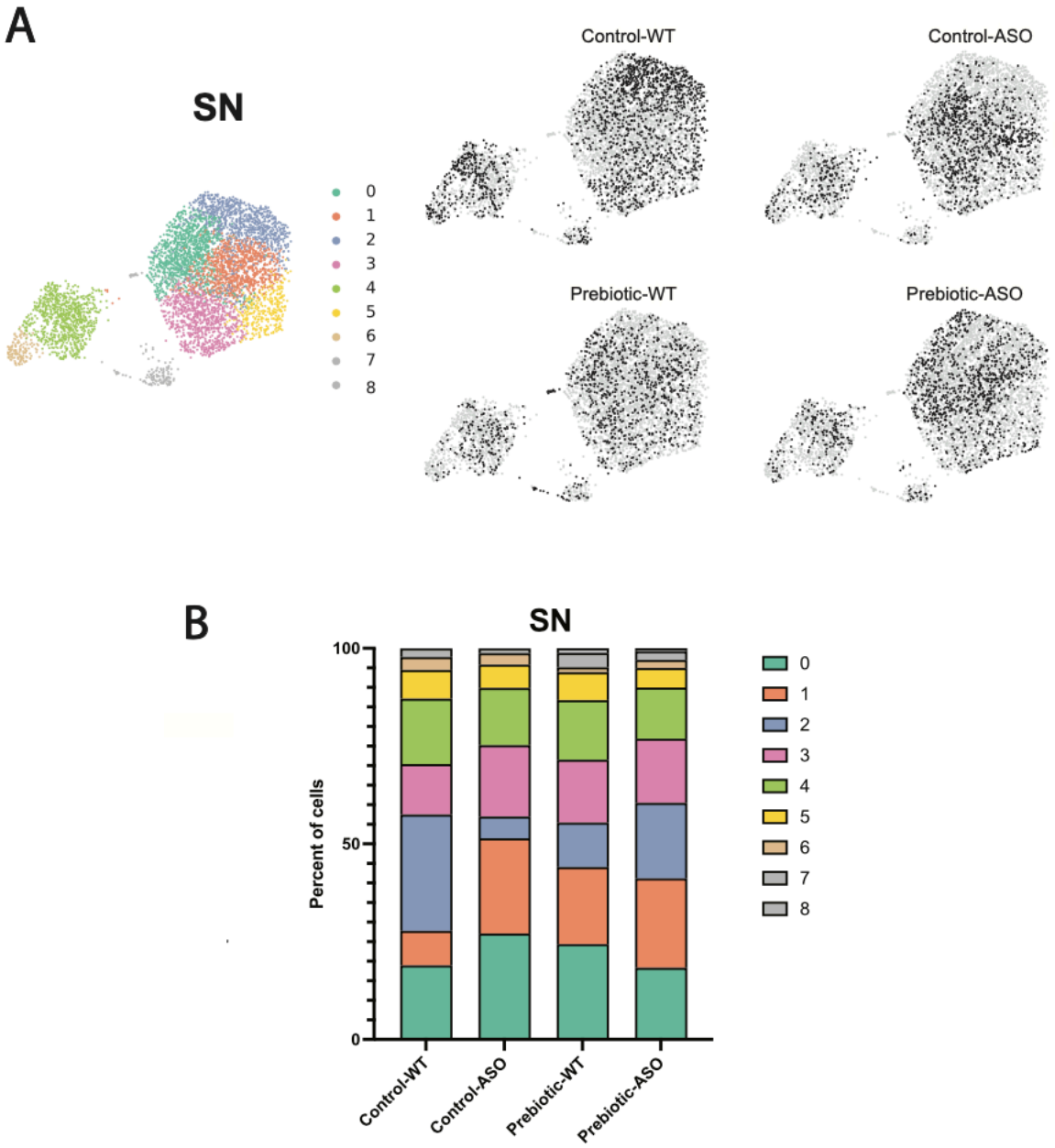


Figure 4: Prebiotic diet alters microglia gene expression

Figure 4C: C) Dot plot showing genes significantly upregulated in control-ASO microglia (relative to control-WT) and significantly downregulated in prebiotic-ASO microglia (relative to control-ASO) in the SN.

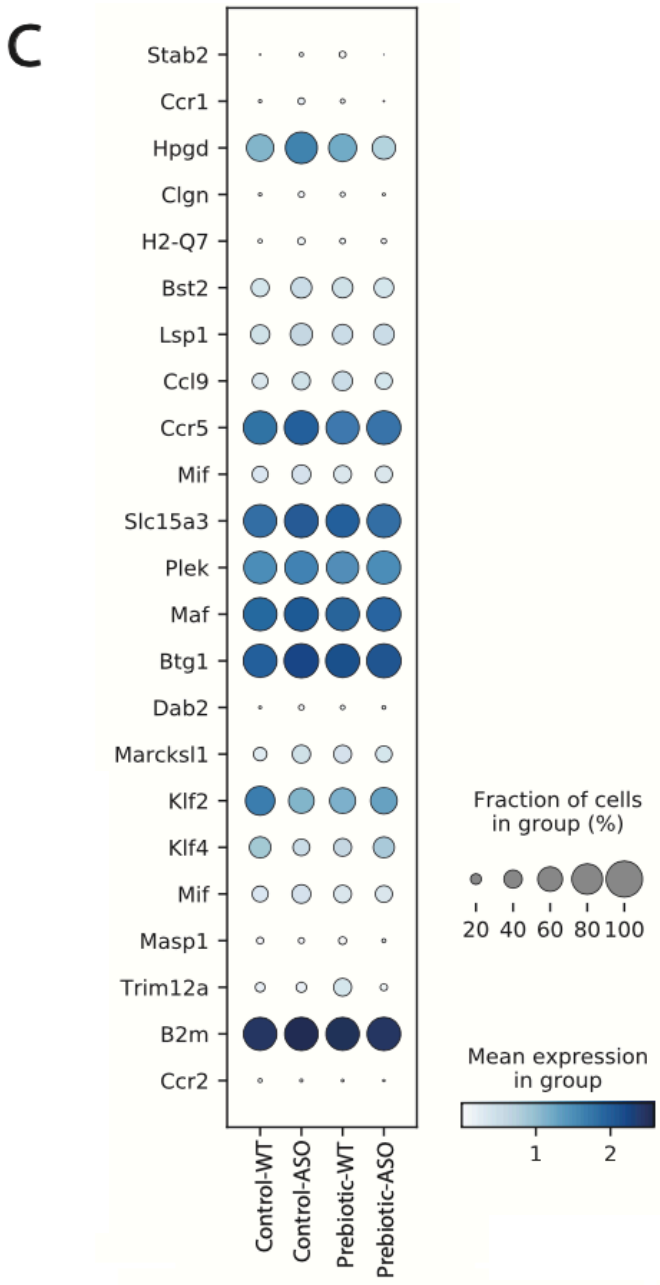
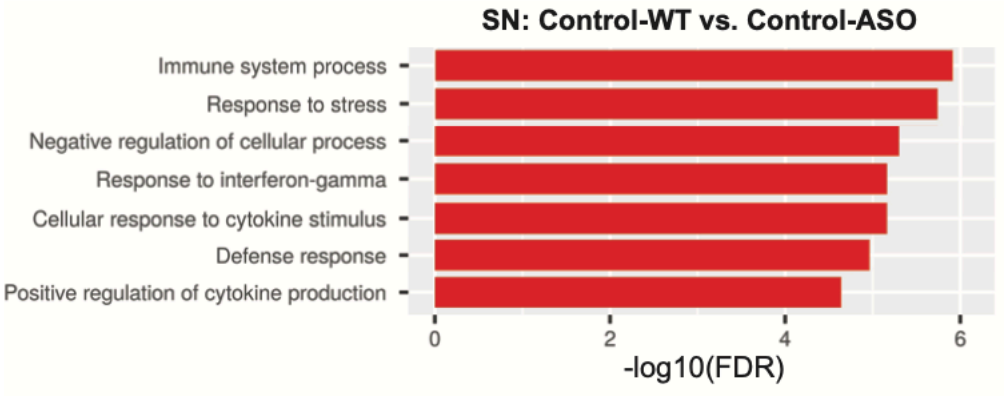


Figure 4: Prebiotic diet alters microglia gene expression

Figure 4D-E: D) Selection of the top 20 significantly enriched pathways among 163 genes upregulated in control-ASO microglia relative to control-WT microglia in the SN. E) Significantly enriched pathways among 156 downregulated genes in prebiotic-ASO microglia relative to control-ASO microglia in the SN.

D



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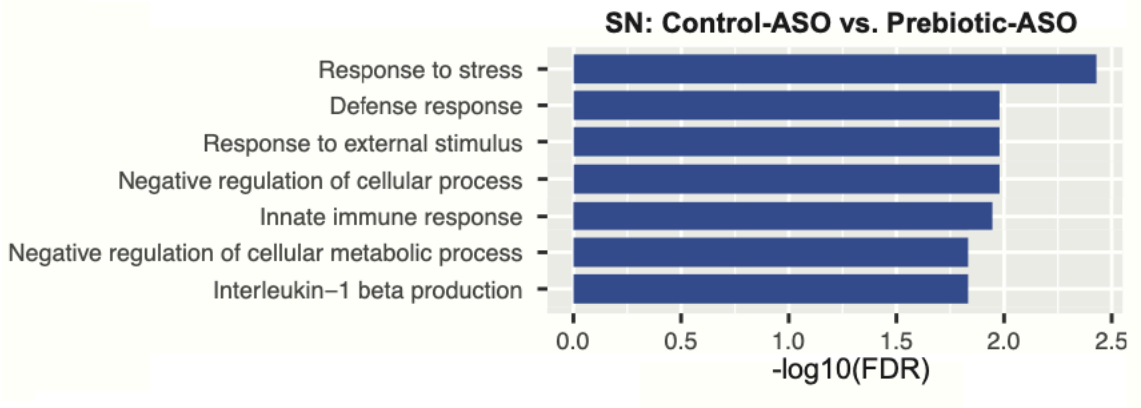
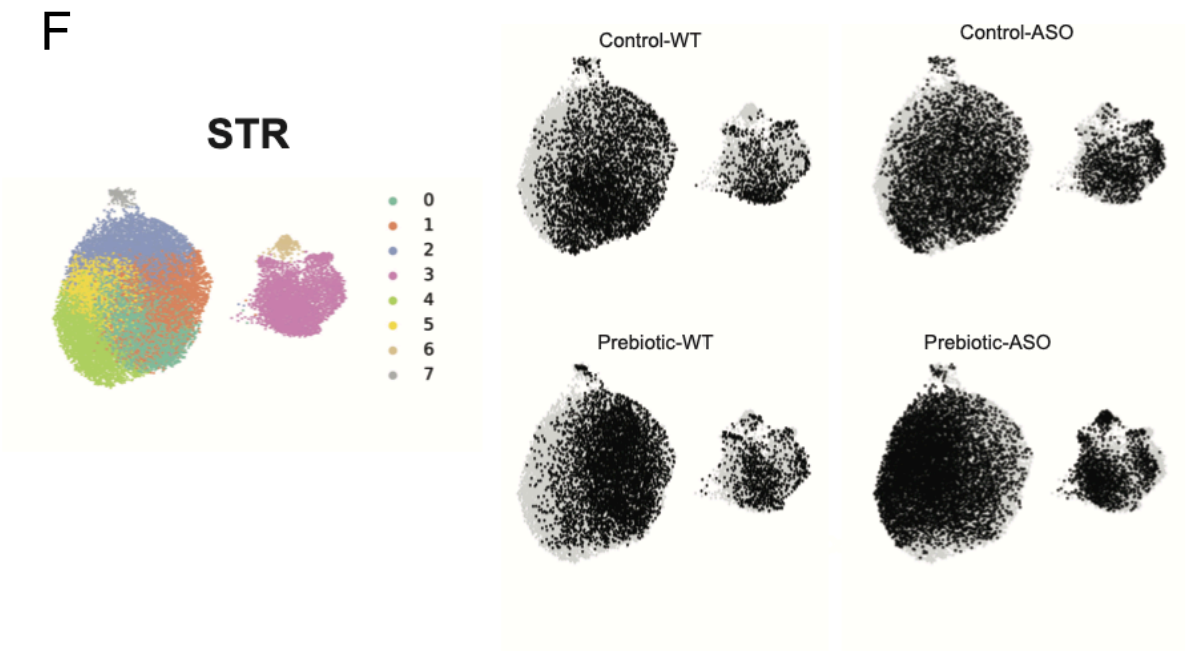


Figure 4: Prebiotic diet alters microglia gene expression

Figure 4F-G: F) UMAP plot of all cells sequenced in the in the STR (n=27152 cells). Right) Distribution of cells in individual samples G) Relative distribution of cells within each cluster in the STR.



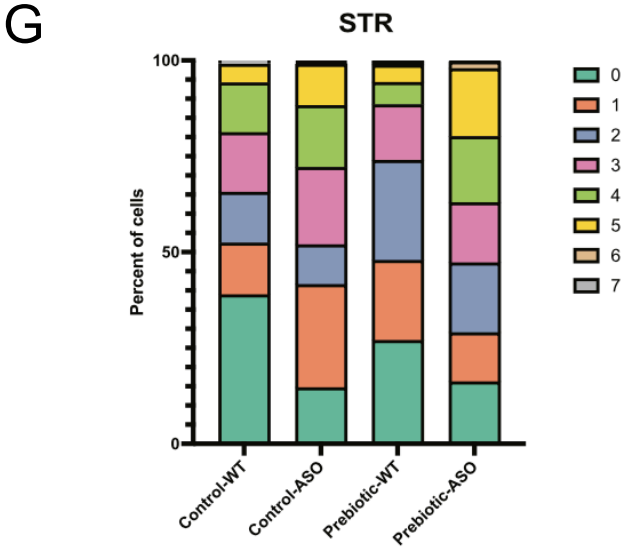


Figure 4: Prebiotic diet alters microglia gene expression

Figure 4H: H) Dot plot showing genes significantly upregulated in control-ASO microglia (relative to control-WT) and significantly downregulated in prebiotic-ASO microglia (relative to control-ASO) in the STR.

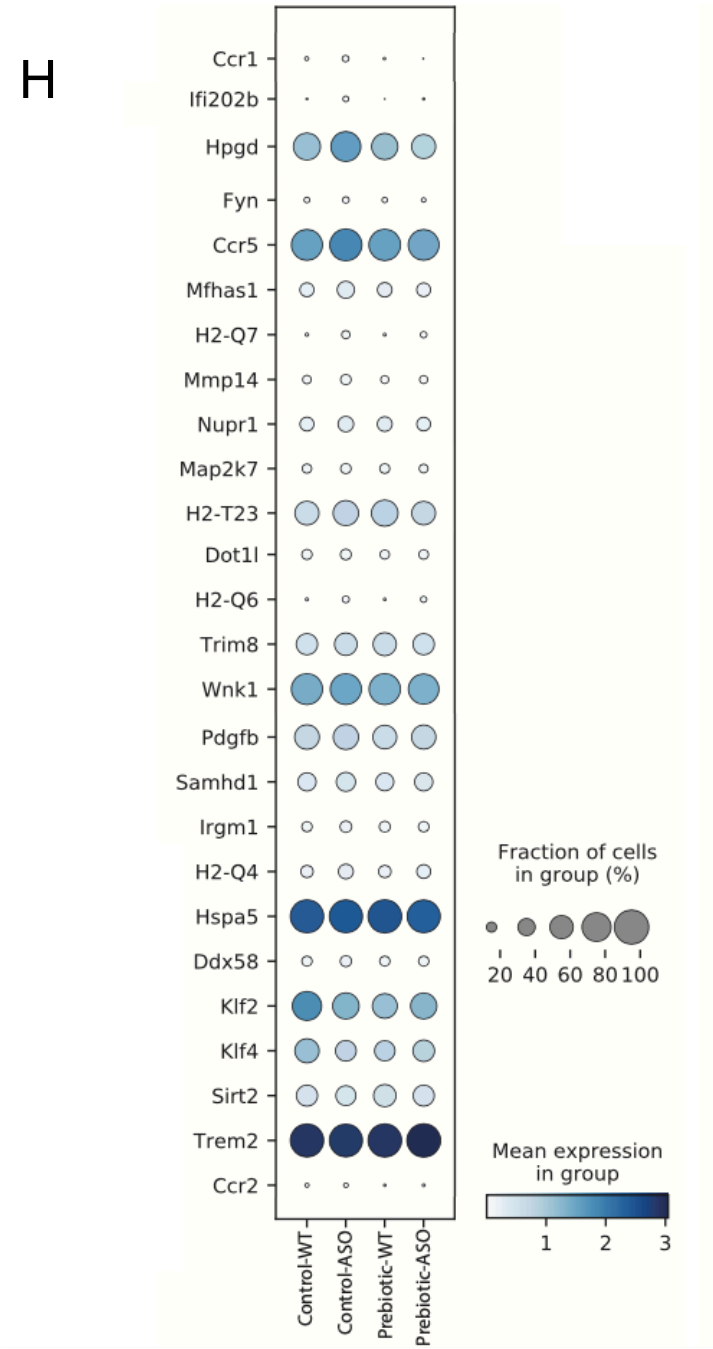


Figure 4: Prebiotic diet alters microglia gene expression

Figure 4I-J: I) Selection of the top 20 significantly enriched pathways among 50 most upregulated genes in control-ASO microglia relative to control-WT microglia in the STR. J) Significantly enriched pathways among 50 most downregulated genes in prebiotic-ASO microglia relative to control-ASO microglia in the STR.

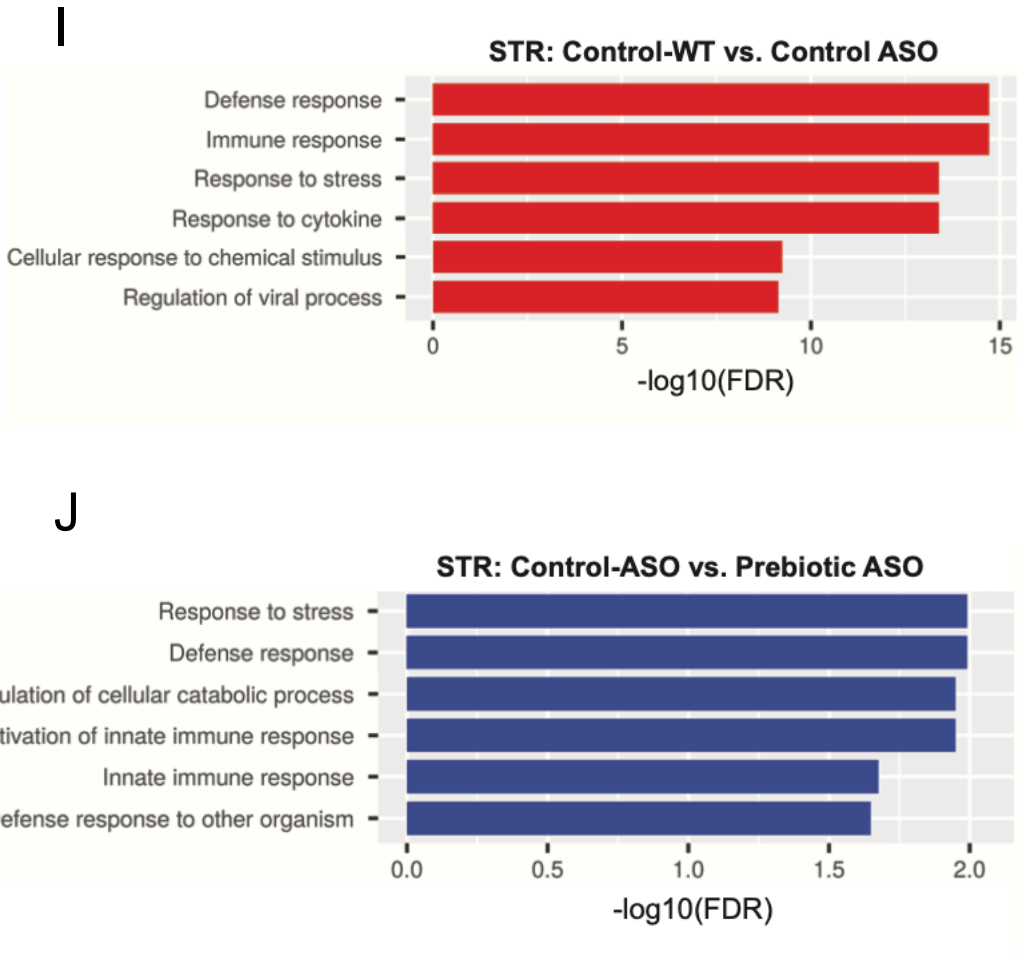
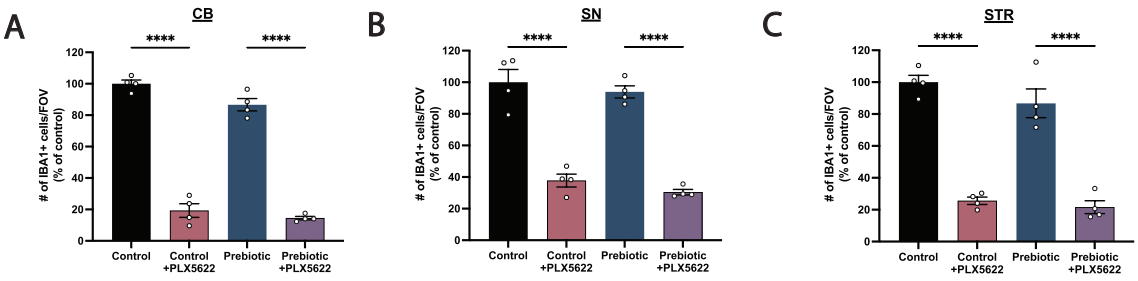


Figure 5: Depletion of microglia blocks beneficial effects of prebiotics on motor performance and α-synuclein aggregation

Figure 5A-C: Number of IBA1+ cells in the A) Cerebellum (n=4/group), B) Substantia Nigra (n=4/group), and C) Striatum (left: # of IBA1+ cells/FOV; right: 20x image of IBA1+ staining in striatum, scale bar: 50 μm). Microglia count data analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



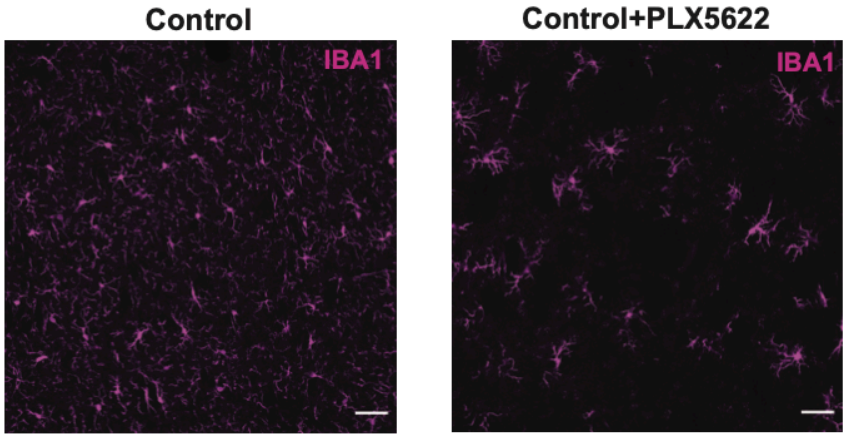


Figure 5: Depletion of microglia blocks beneficial effects of prebiotics on motor performance and α-synuclein aggregation

Figure 5D-F: Motor performance in the D) Pole test E) Beam traversal-time to cross and F) Beam traversal-errors per step (n=12-21/group). Motor data derived from five independent cohorts. Motor data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001

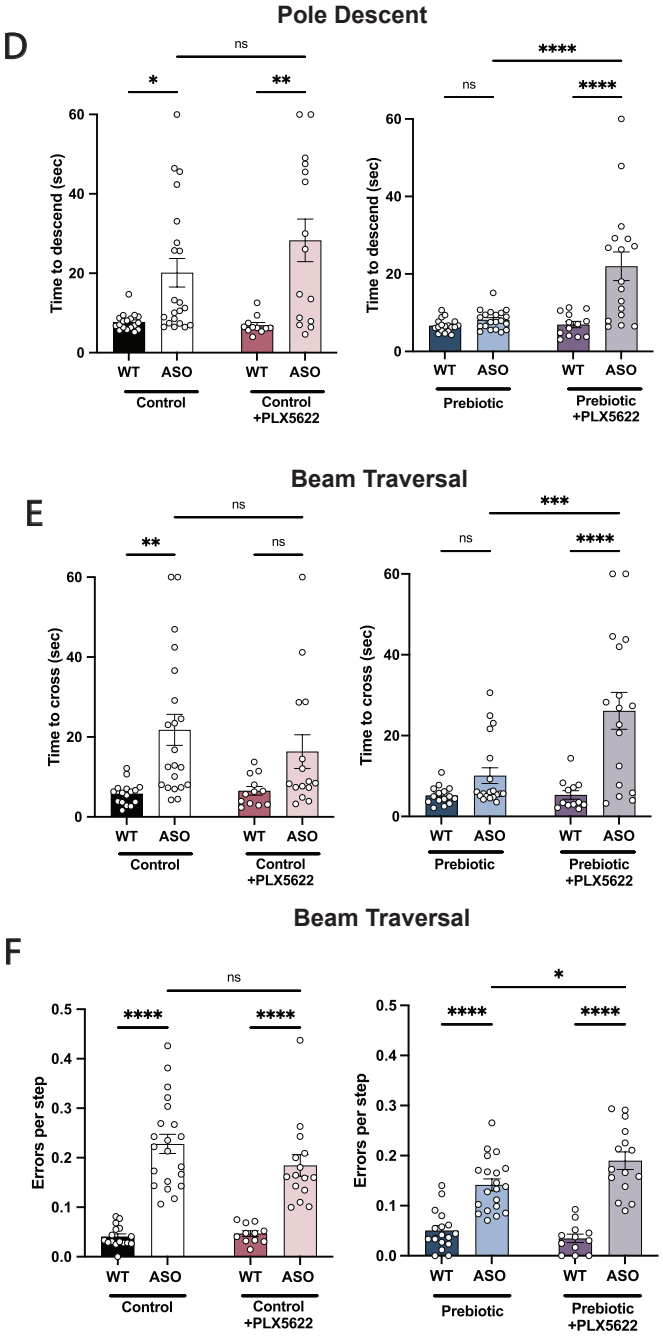


Figure 5: Depletion of microglia blocks beneficial effects of prebiotics on motor performance and α-synuclein aggregation

Figure 5G: Aggregated α-synuclein measured by dot blot in the G) Substantia Nigra (n=6-10/group). αSyn data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001

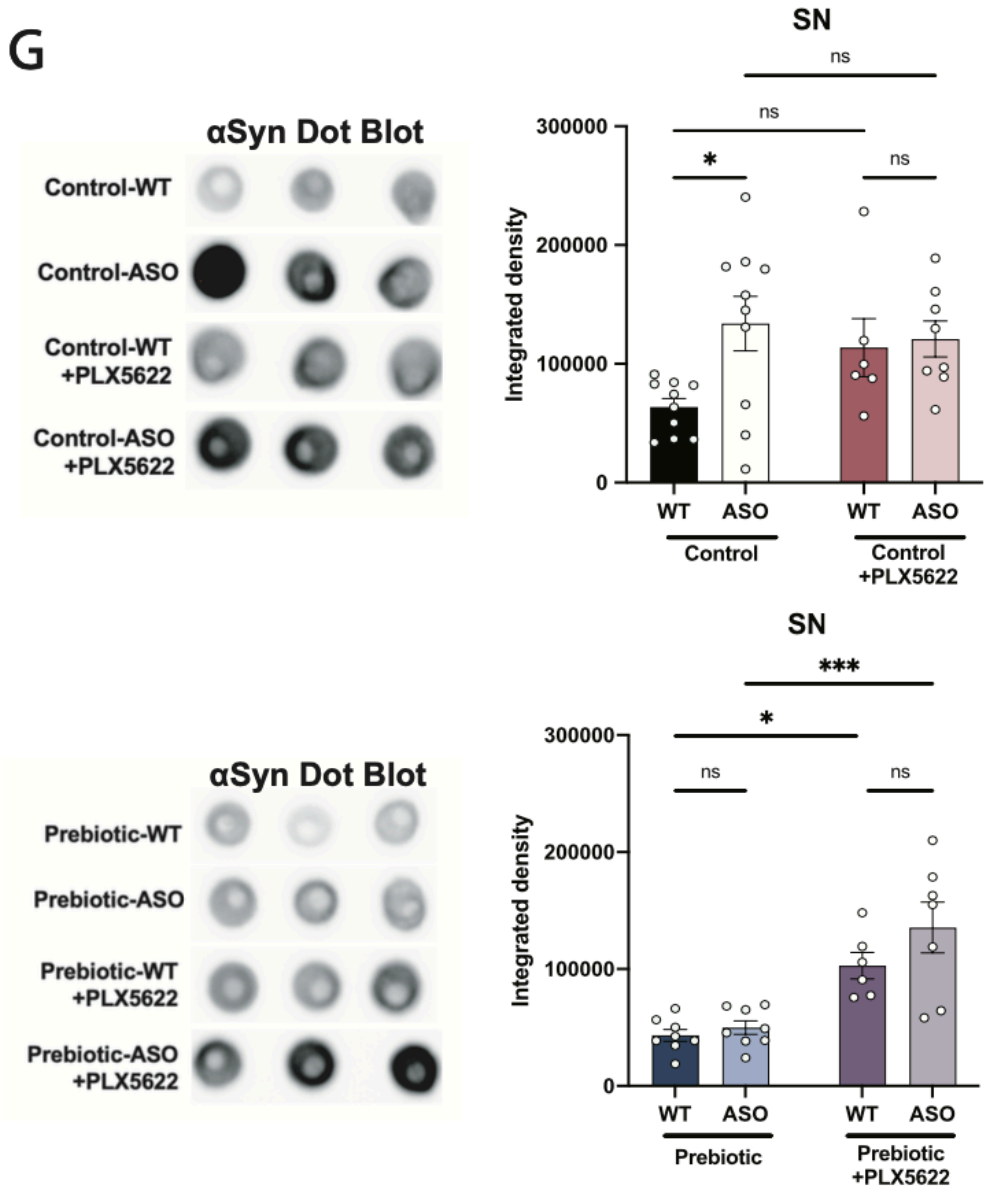
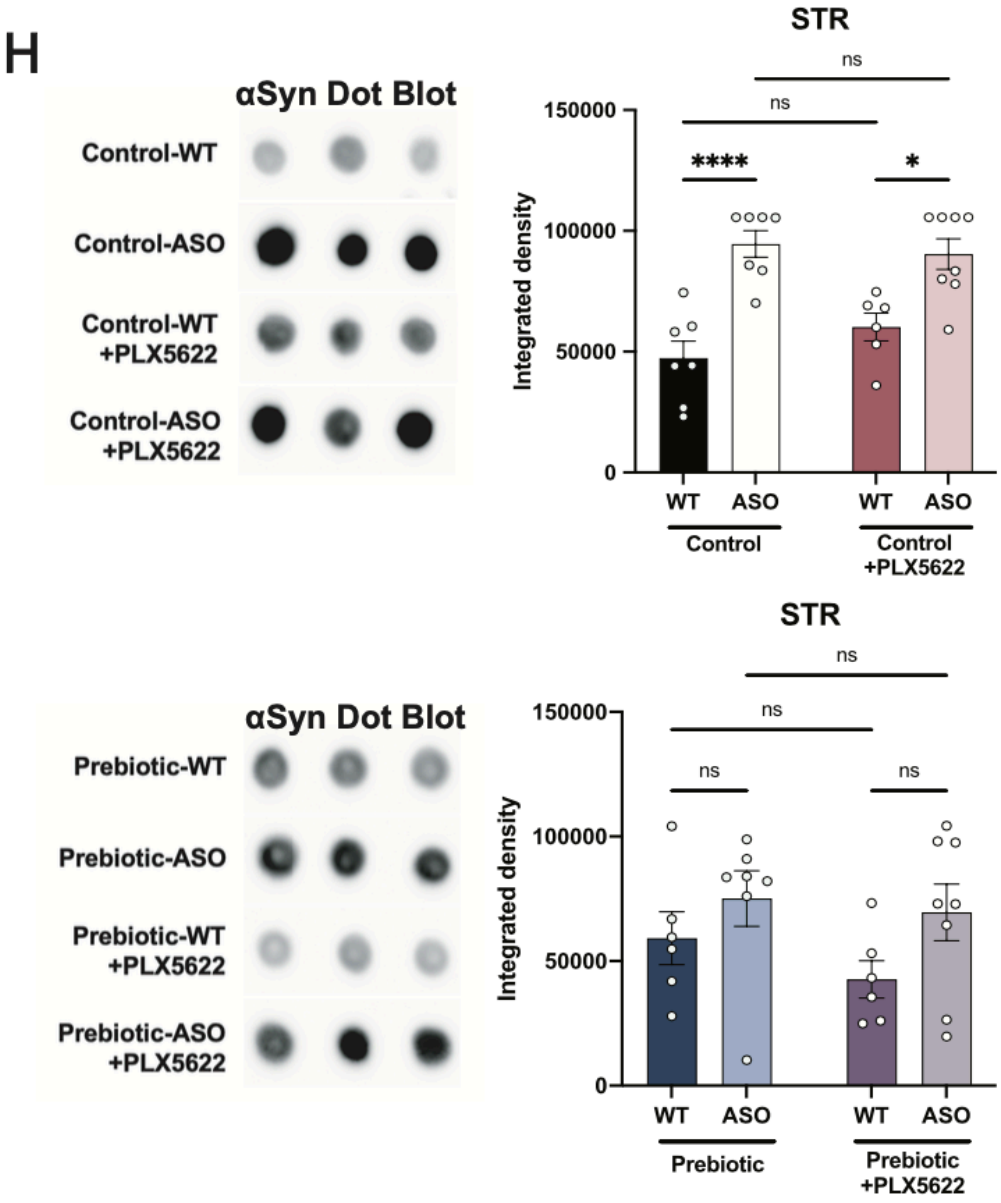


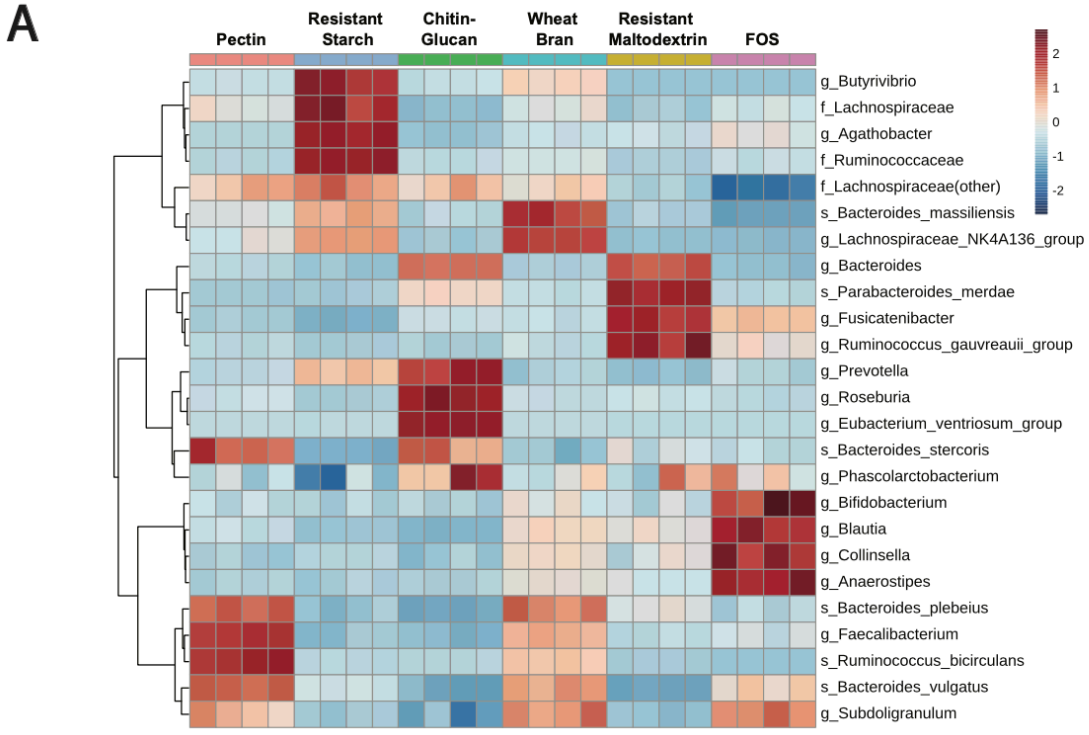
Figure 5: Depletion of microglia blocks beneficial effects of prebiotics on motor performance and α-synuclein aggregation

Figure 5H: Aggregated α-synuclein measured by dot blot in the H) Striatum (n=6-8/group). αSyn data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



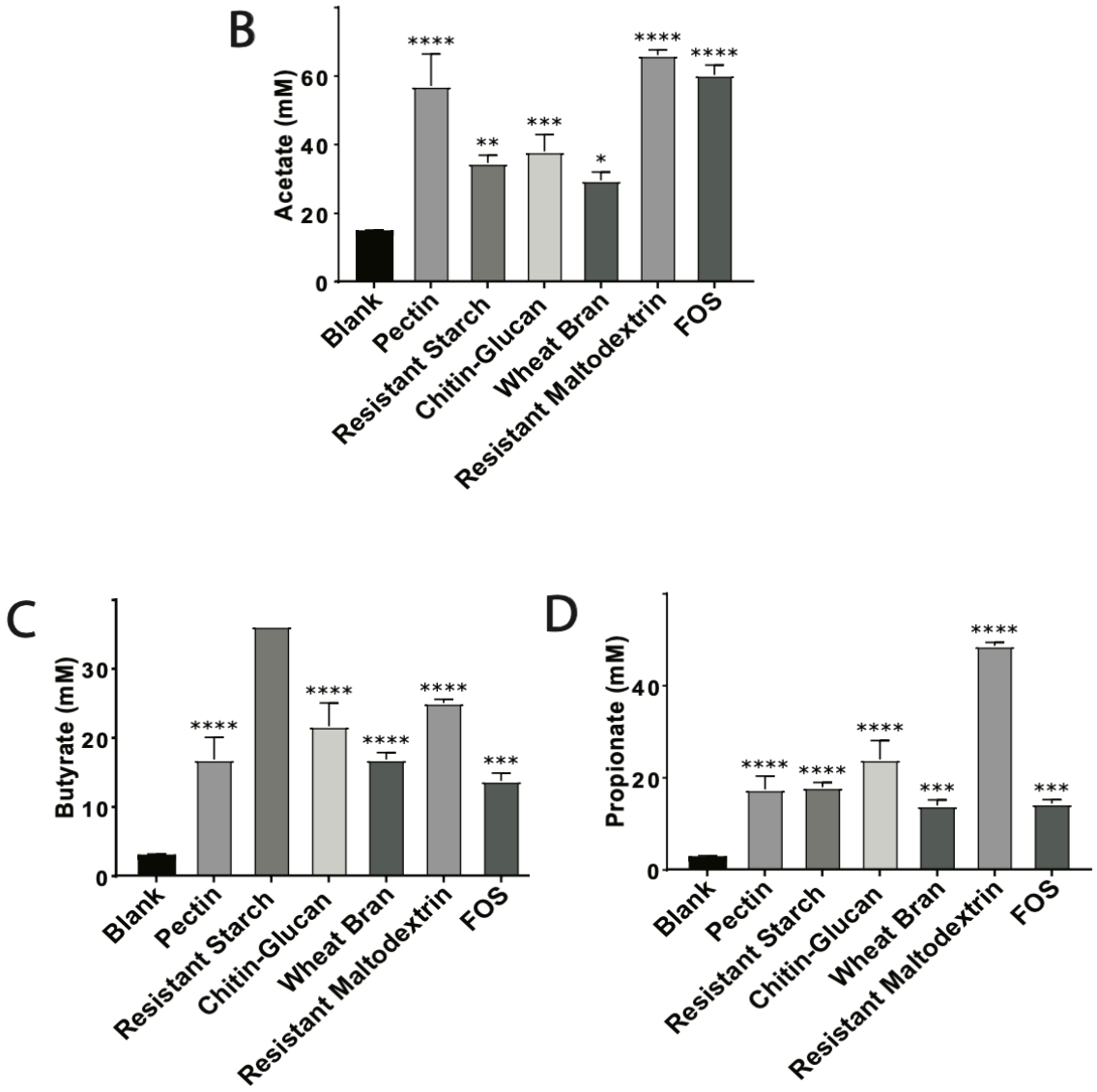
Supplement Figure 1: Effect of dietary fibers that compose the three prebiotic mixtures on gut microbial community and metabolic function *in vitro*

Supplement Figure 1A: A) Hierarchical clustering of the 25 most abundant genera after 24 h *in vitro* fecal fermentation using a pooled human gut microbiota community as previously described (Cantu-Jungles et al., 2018). Clusters of taxa were associated with fiber types. Hierarchical clustering was performed using Euclidean distances and the Ward algorithm.



Supplement Figure 1: Effect of dietary fibers that compose the three prebiotic mixtures on gut microbial community and metabolic function *in vitro*

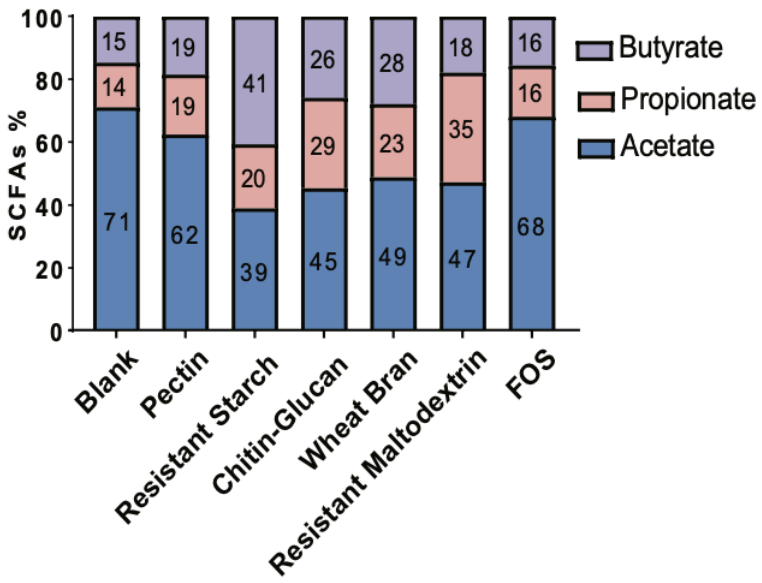
Supplement Figure 1B-D: SCFA concentration (mM) in fecal slurries. B) Acetate C) Butyrate and C) Propionate. Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Statistical differences from the blank are marked by *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



Supplement Figure 1: Effect of dietary fibers that compose the three prebiotic mixtures on gut microbial community and metabolic function *in vitro*

Supplement Figure 1E-F: E) Relative proportion of each SCFA produced in the 24 h *in vitro* fecal fermentation, showing association with fiber types. F) Fiber composition in Prebiotic Diet 1-3.

E

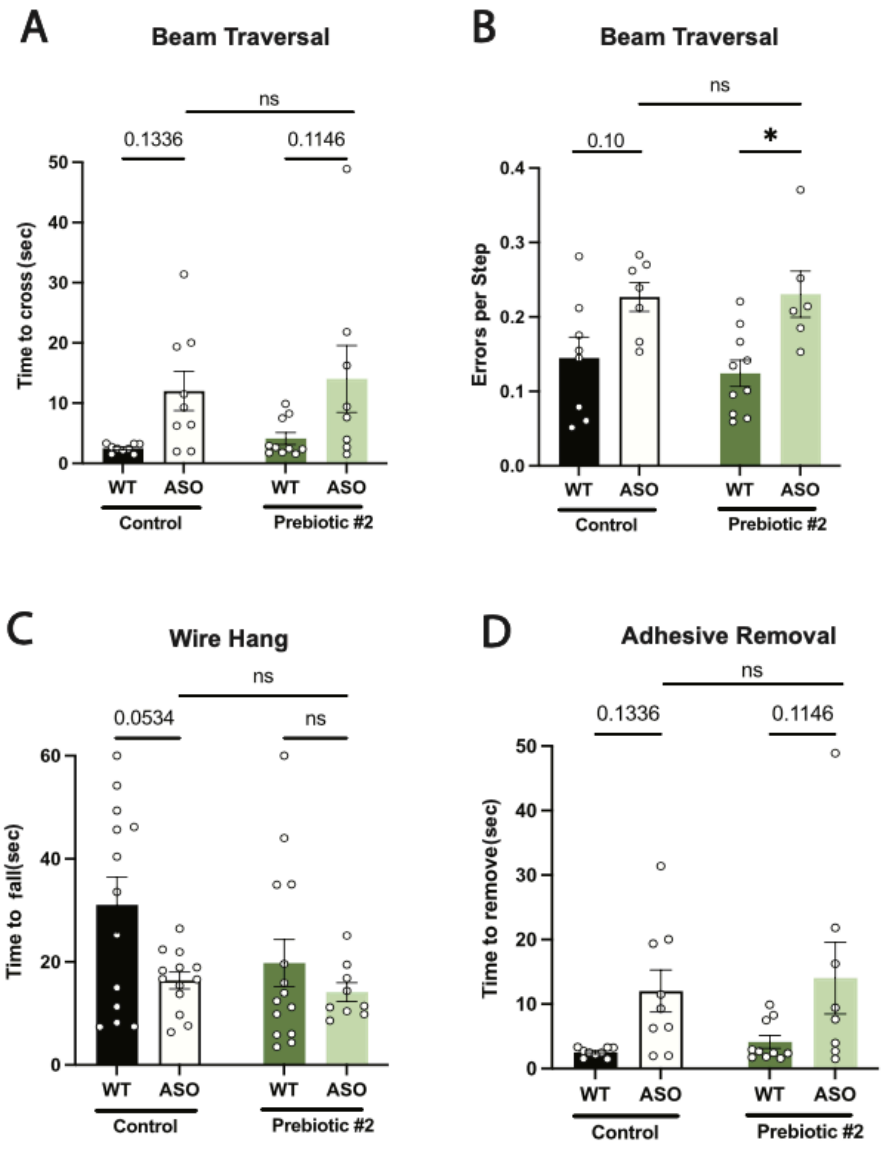


F

Dietary Fiber	Prebiotic #1	Prebiotic #2	Prebiotic #3
Pectin	-	-	25%
Resistant Starch	-	50%	-
Chitin-Glucan	-	50%	-
Wheat Bran	50%	-	-
Resistant Maltodextrin	50%	-	50%
FOS	-	-	25%

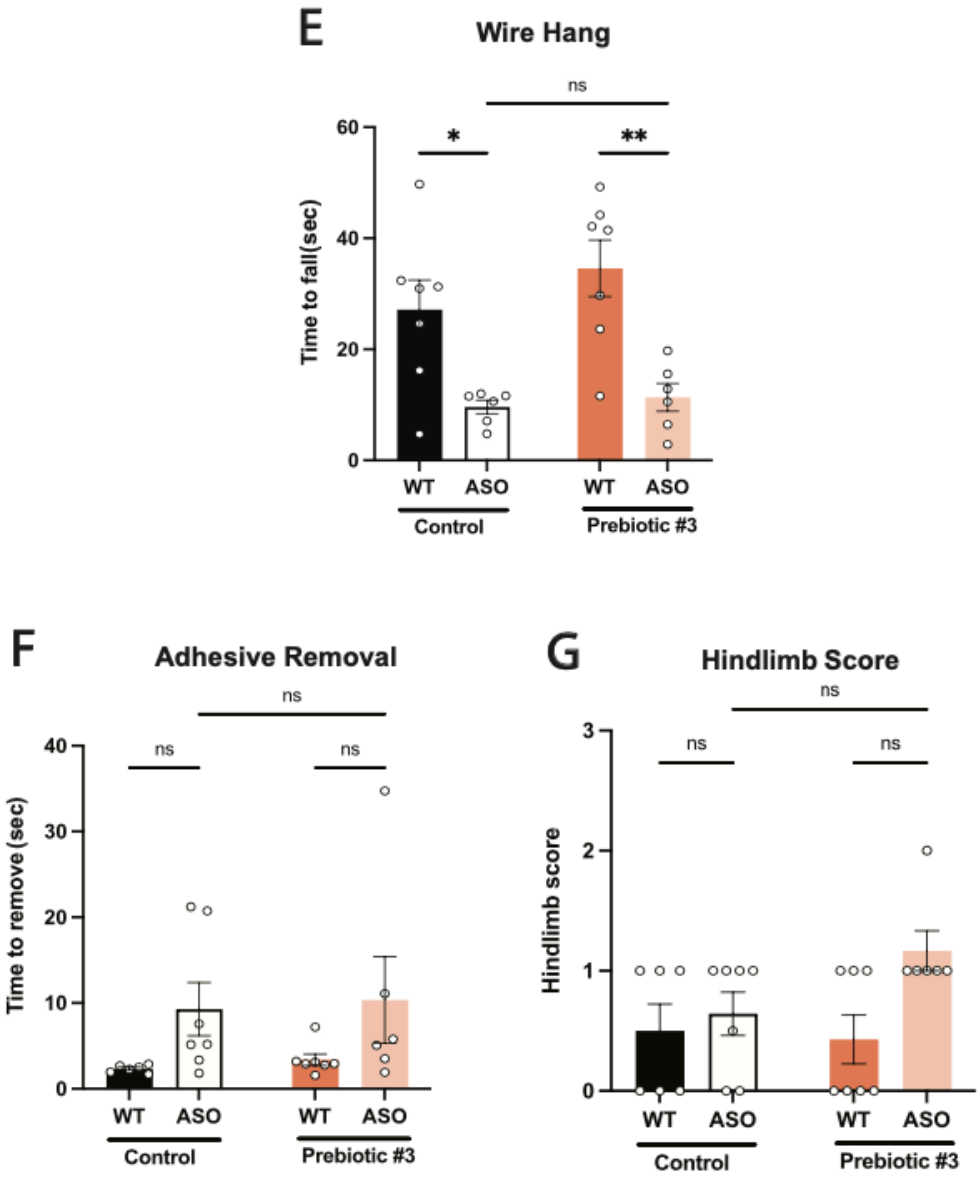
Supplement Figure 2: Motor behavior data from mice fed Prebiotic #2 and Prebiotic #3 diet

Supplement Figure 2A-D: Motor behavior data from Prebiotic #2 and Prebiotic #3. (A-D) Motor behavior data for Prebiotic #2. A) Beam-time to cross B) Beam-errors per step C) Wire hang D) Adhesive removal (n=6-14/group). Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



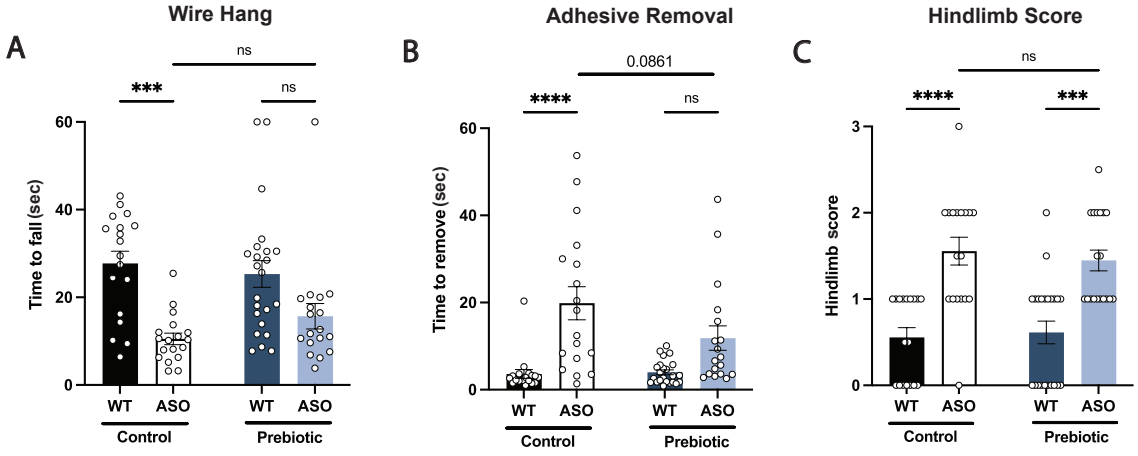
Supplement Figure 2: Motor behavior data from mice fed Prebiotic #2 and Prebiotic #3 diet

Supplement Figure 2E-G: (E-G) Motor behavior data for Prebiotic #3. E) Wire hang F) Adhesive removal G) Hindlimb score (n=6-7/group). Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Hindlimb score data analyzed by Kruskal-Wallis test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



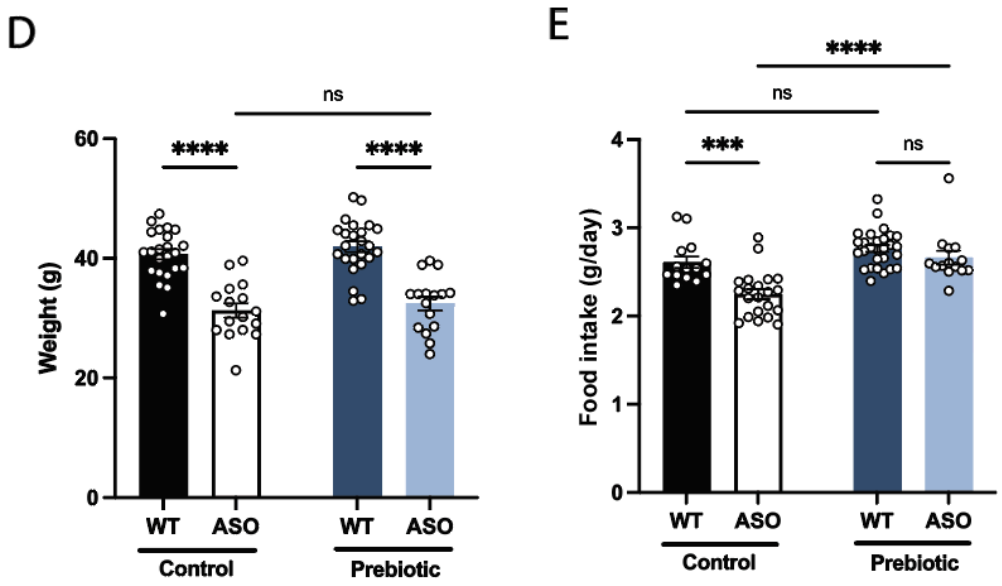
Supplement Figure 3: Prebiotic diet does not improve motor performance on select motor tests and has no effect on body weight.

Supplement Figure 3A-C: Motor symptoms at 22 weeks in A) Wire hang, B) Adhesive removal, and C) Hindlimb score test (n=18-24.group). Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Hindlimb score data analyzed by Kruskal-Wallis test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



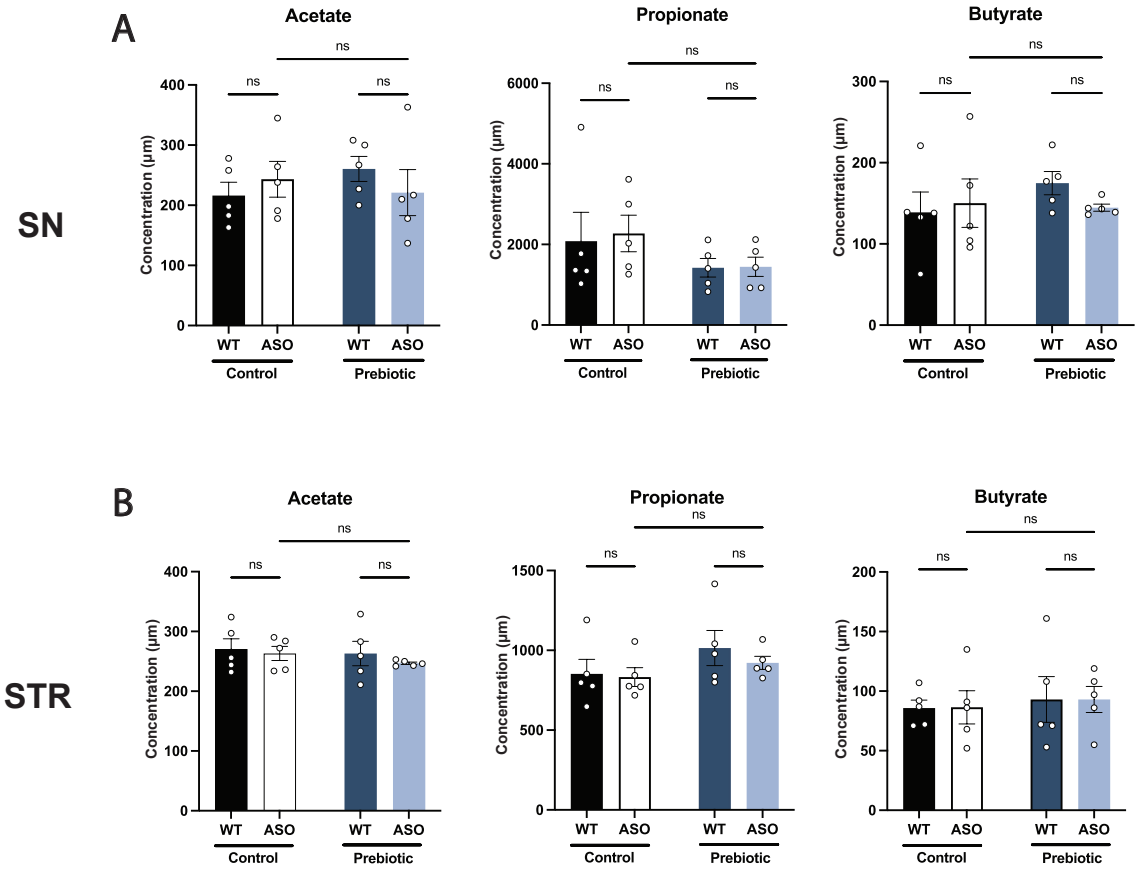
Supplement Figure 3: Prebiotic diet does not improve motor performance on select motor tests and has no effect on body weight.

Supplement Figure 3D-E: D) Mouse weight at 22 weeks (n=16-24/group). E) Food intake per mouse recorded over the course of experiment. Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



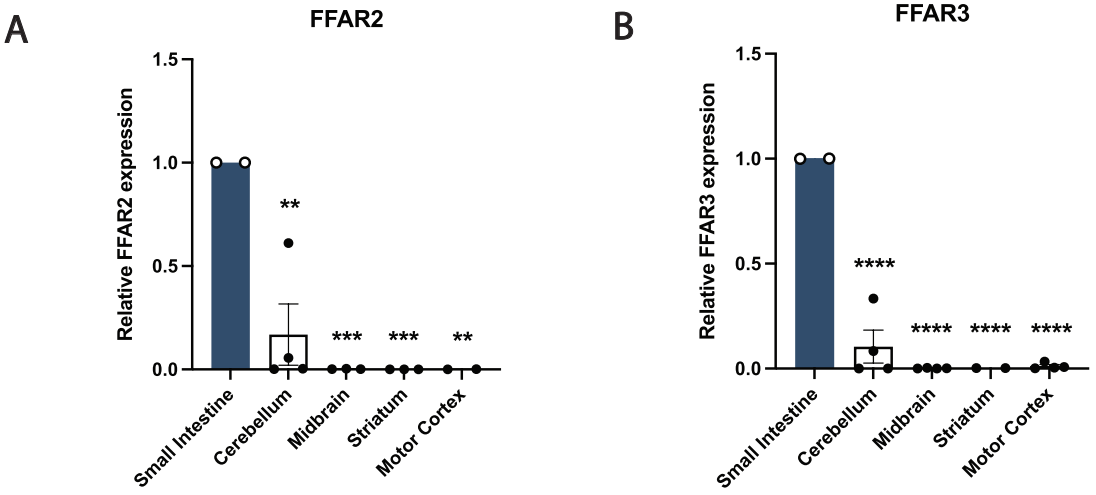
Supplement Figure 4: Prebiotics do not change SCFA levels in the brain

Supplement Figure 4A-B: Concentration (μM) of acetate, propionate, and butyrate measured by UHP-LC in the A) Substantia Nigra (n=5/group) and B) Striatum (n=5/group). Each point represents data from one individual mouse. SCFA data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



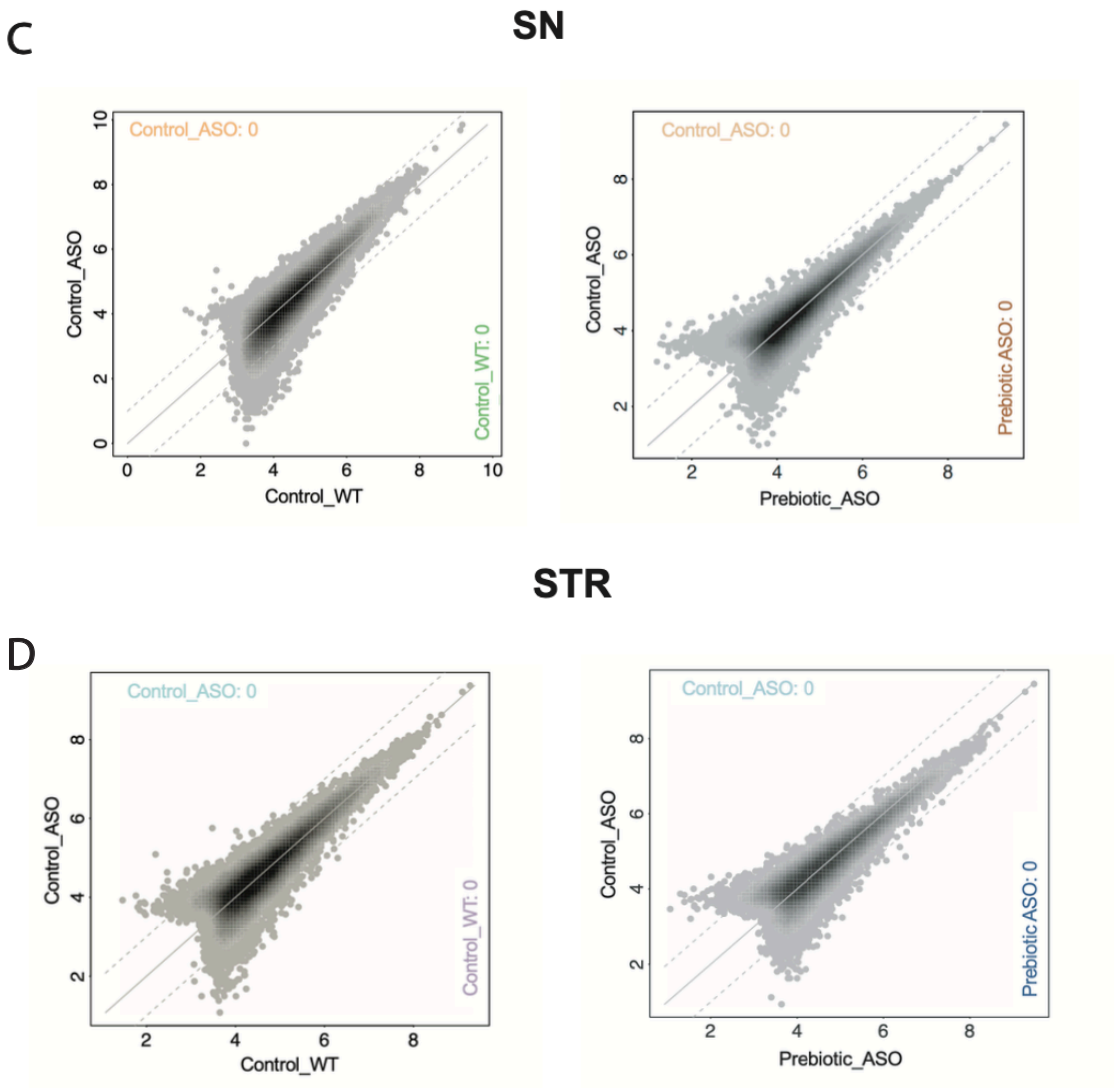
Supplement Figure 5: Measurement of FFAR2/3 in brain and GI tissue and epigenetic characterization of prebiotic-fed mice

Supplement Figure 5A-B: qPCR measurement of A) FFAR2 and B) FFAR 3 in small intestine, cerebellum, midbrain, striatum and motor cortex reveals little to no expression of either receptor in the brain (n=2-4). FFAR2/3 qPCR data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



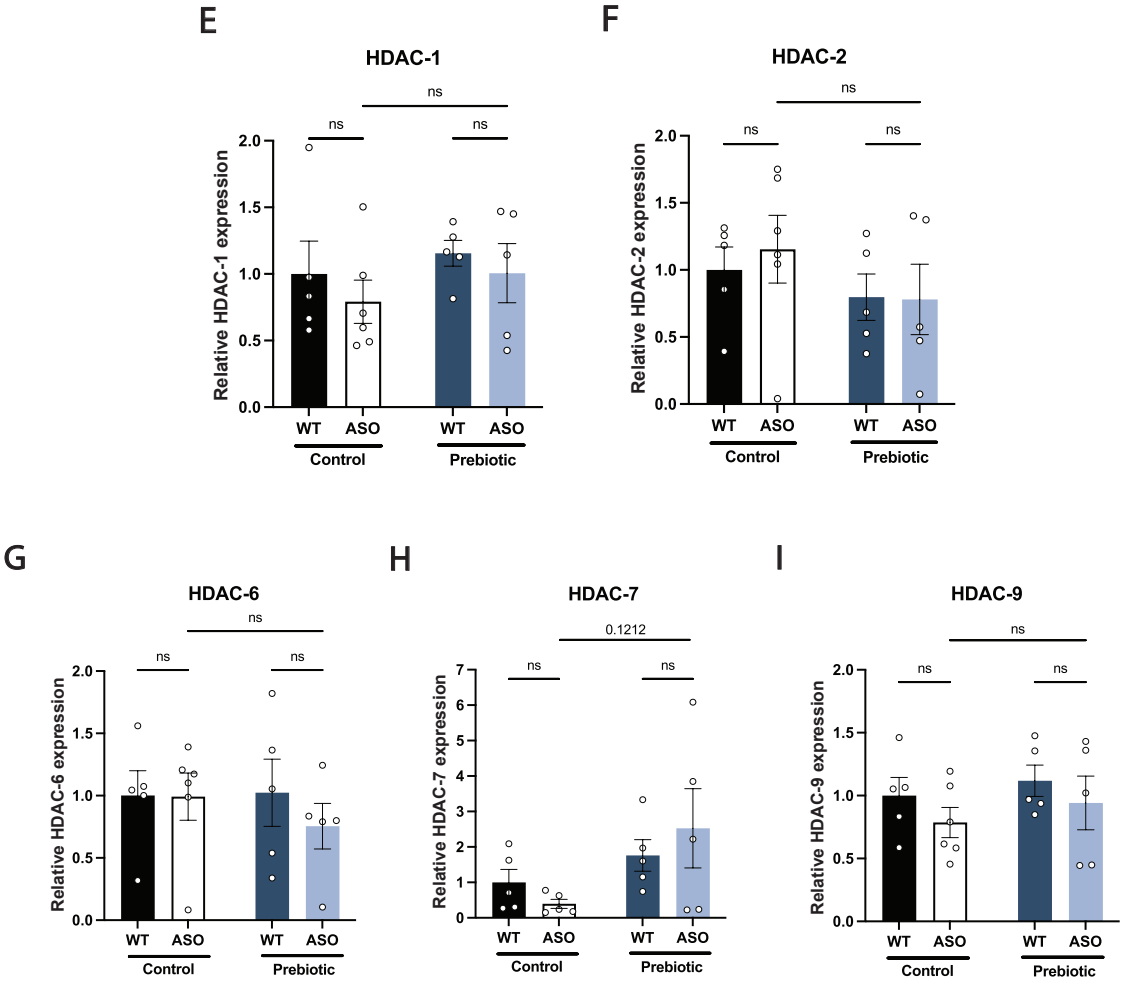
Supplement Figure 5: Measurement of FFAR2/3 in brain and GI tissue and epigenetic characterization of prebiotic-fed mice

Supplement Figure 5C-D: ATAC-seq of purified microglia in the C) Substantia Nigra and D) Striatum.



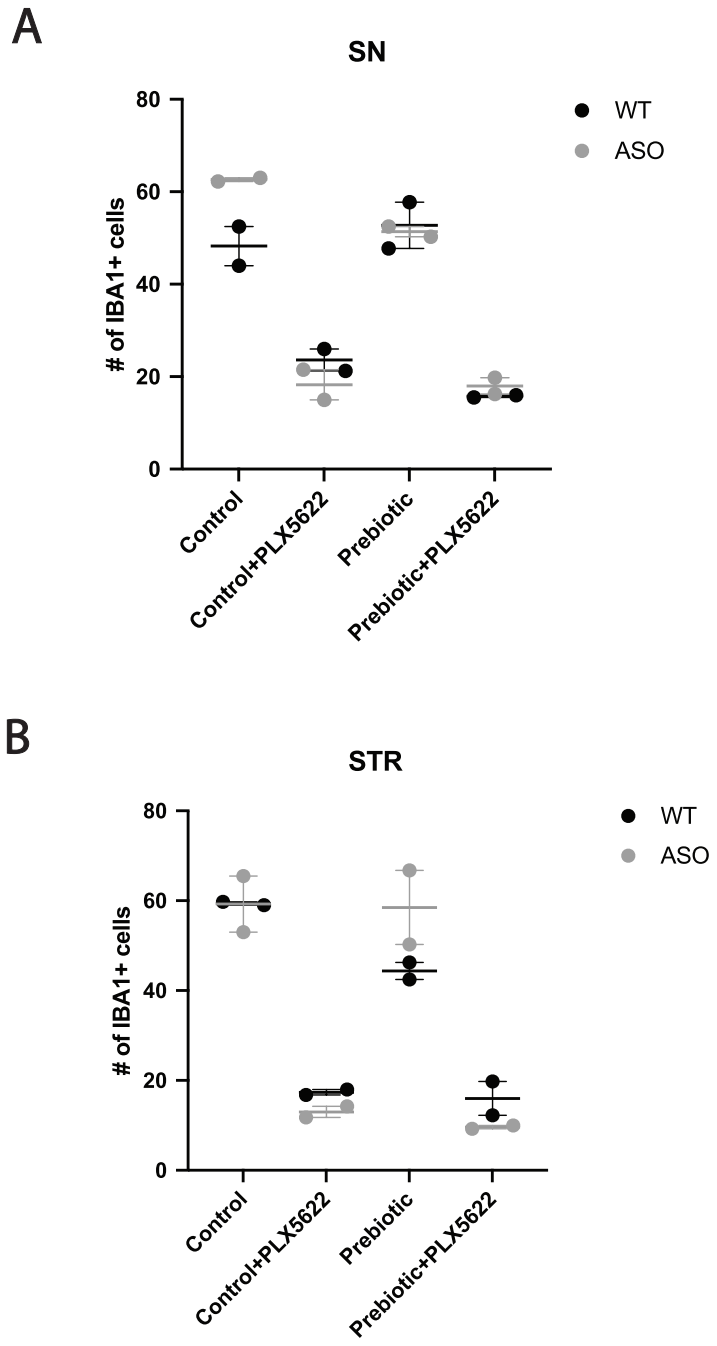
Supplement Figure 5: Measurement of FFAR2/3 in brain and GI tissue and epigenetic characterization of prebiotic-fed mice

Supplement Figure 5E-I: qPCR measurement of E) HDAC-1, F) HDAC-2, G) HDAC-6, H) HDAC-7, and I) HDAC-9 in the striatum (n=5-6). HDAC qPCR data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



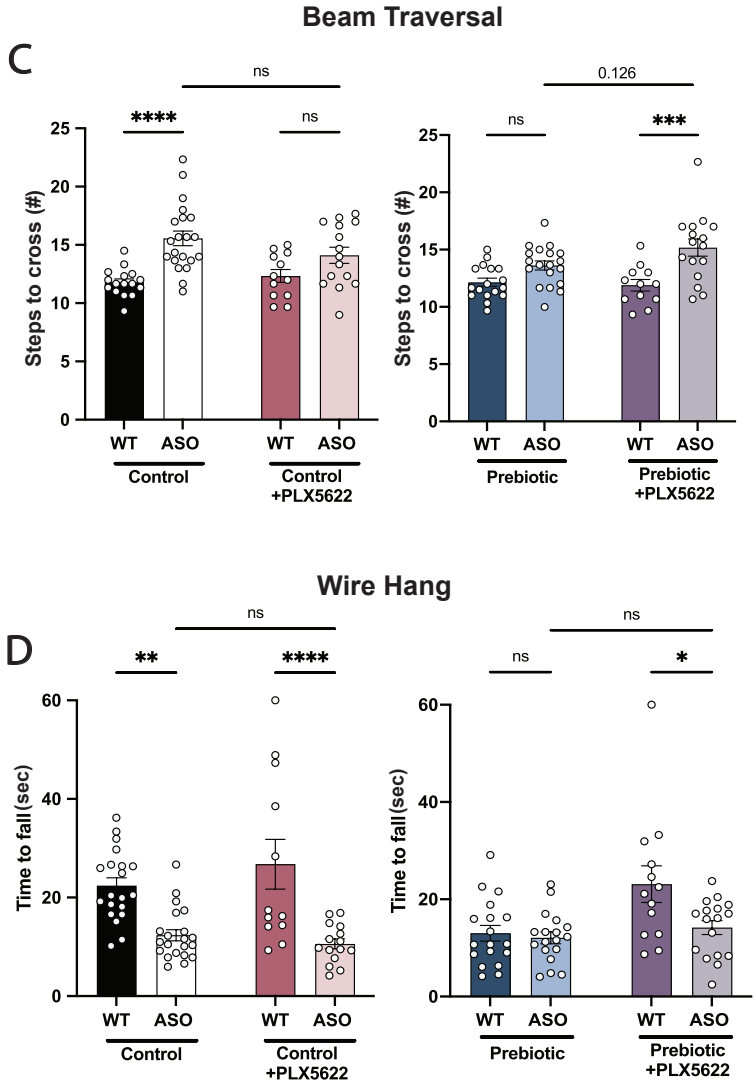
Supplement Figure 6: Additional characterization of PLX5622 treatment

Supplement Figure 6A-B: IBA1+ cell count in the A) Substantia Nigra or B) Striatum (n=2/group).



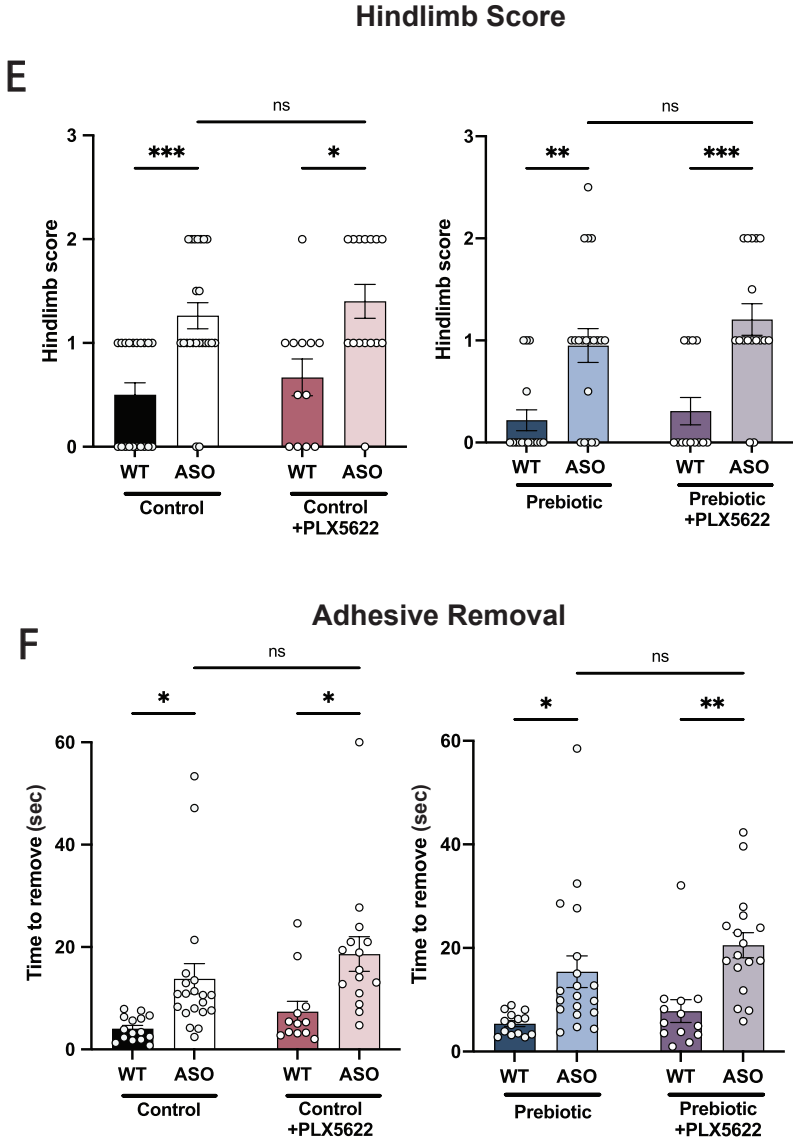
Supplement Figure 6: Additional characterization of PLX5622 treatment

Supplement Figure 6C-D: Motor performance in the C) Beam-number of steps or D) Wire hang test. (n=9-15/group). Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



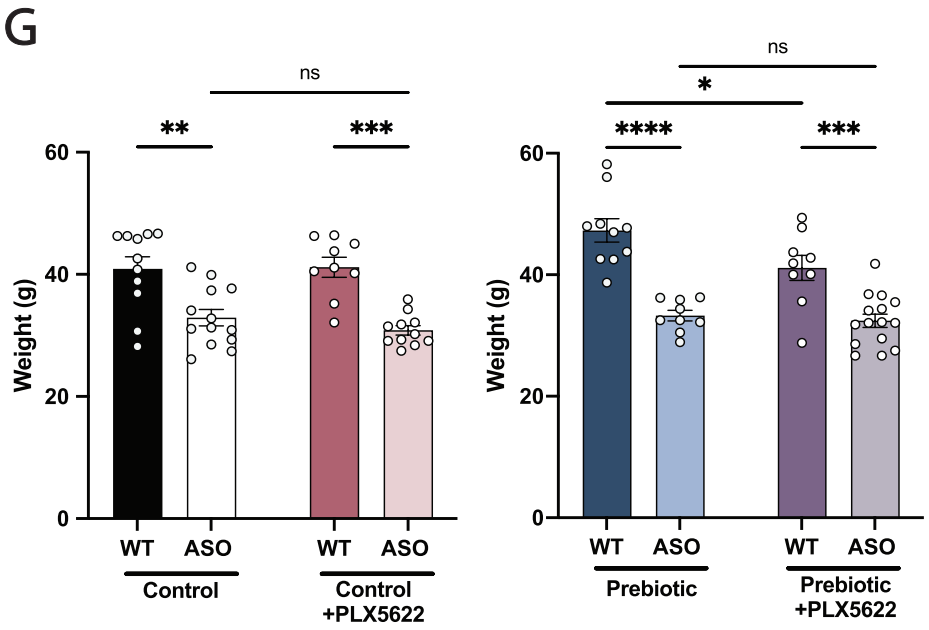
Supplement Figure 6: Additional characterization of PLX5622 treatment

Supplement Figure 6E-F: Motor performance in the E) Hindlimb score or F) Adhesive removal test (n=12-23/group). Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



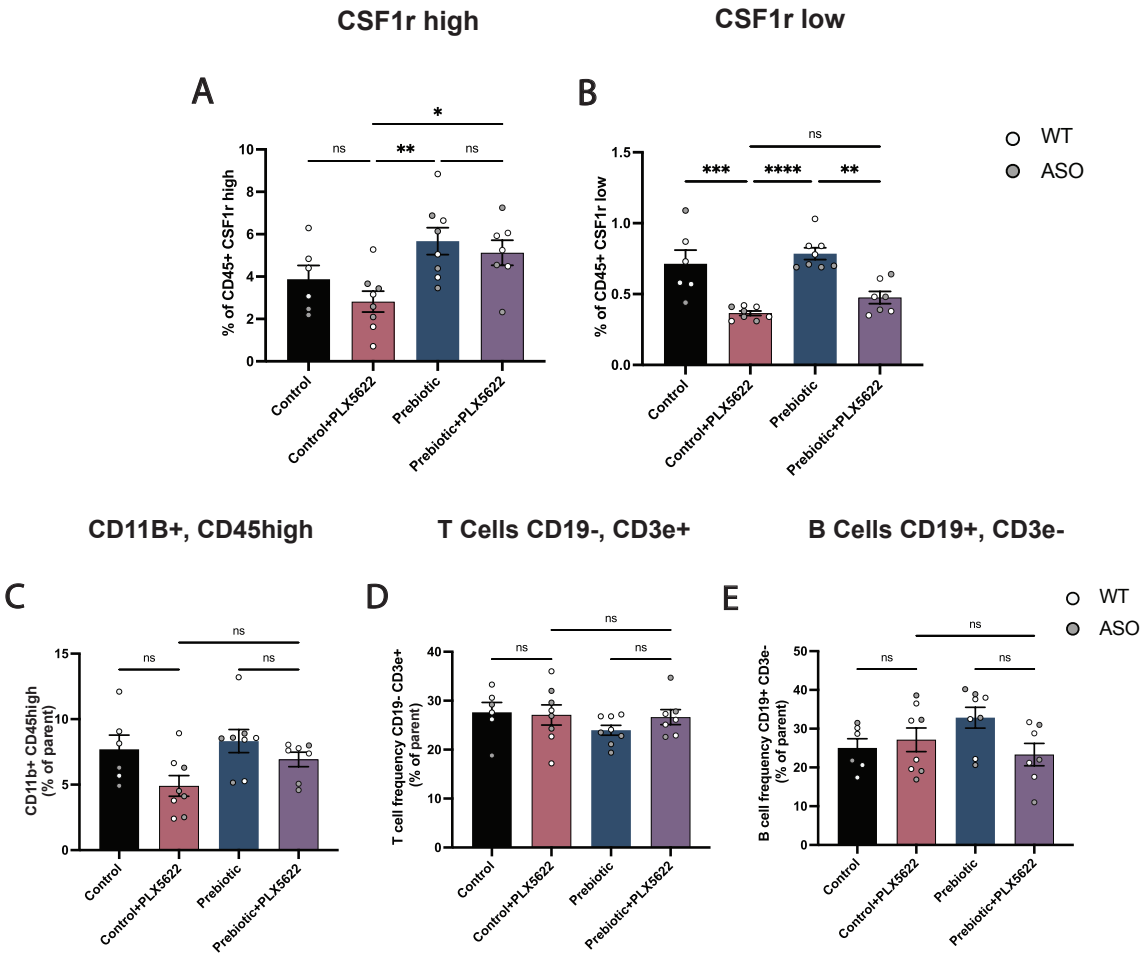
Supplement Figure 6: Additional characterization of PLX5622 treatment

Supplement Figure 6G: G) Mouse weight at 22 weeks (n=9-15/group). Data analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



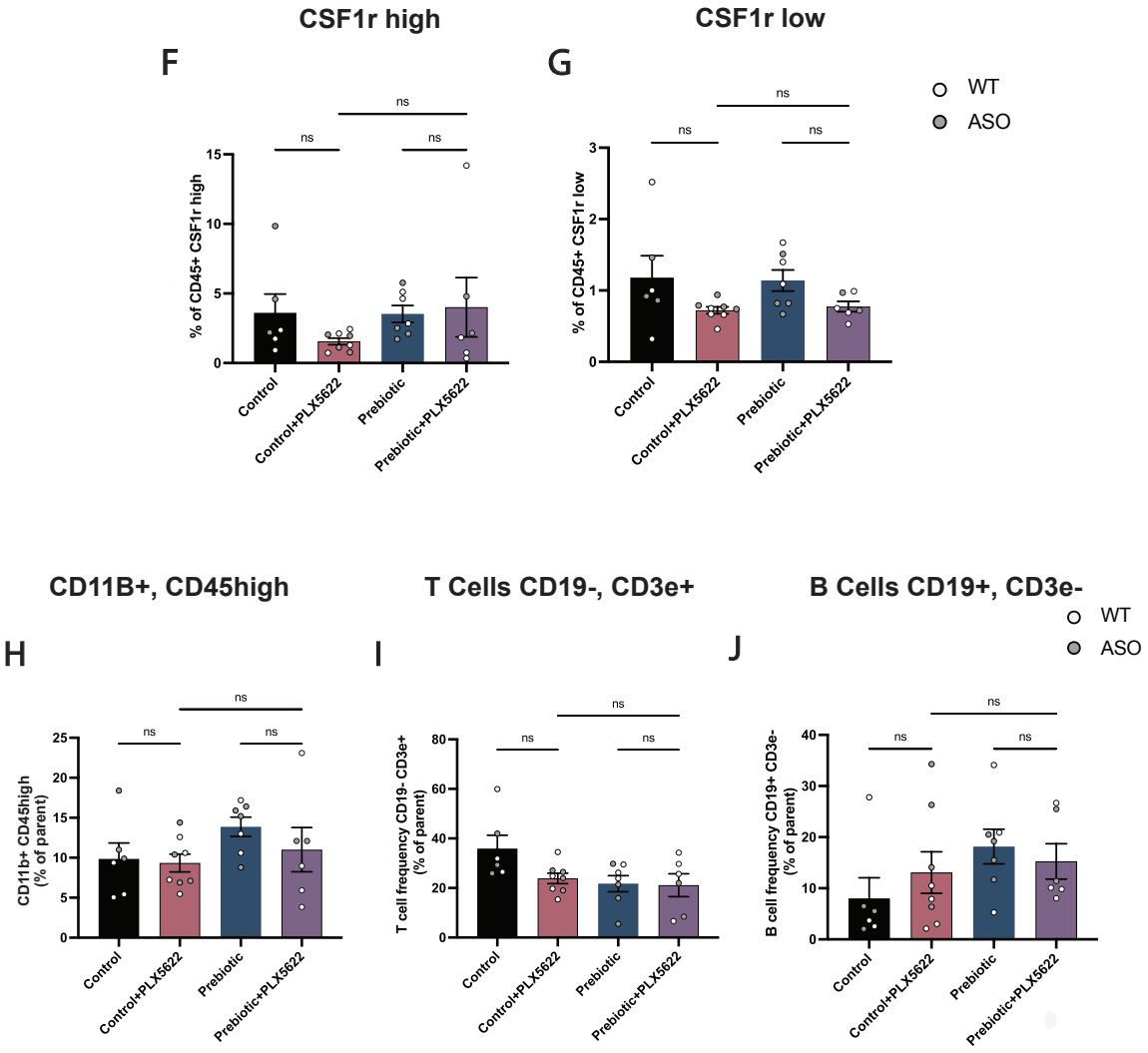
Supplement Figure 7: Immune cell characterization in small intestine, large intestine, and spleen of PLX5622-treated mice

Supplement Figure 7A-E: Large intestinal quantification of A) CD45, CSF1r+ high cells B) CD45+, CSF1r low cells, C) CD11b+, CD45high cells, D) T Cell frequency (CD19-, CD3e+), E) B Cell frequency (CD19+, CD3e-) (n=6-8/group).Each point represents data from an individual mouse. White data points represent WT mice and grey data points represent ASO mice. Data is combined from three independent experiments. Data analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



Supplement Figure 7: Immune cell characterization in small intestine, large intestine, and spleen of PLX5622-treated mice

Supplement Figure 7F-J: Small Intestine: F) CD45, CSF1r+ high cells G) CD45+, CSF1r low cells, H) CD11b+, CD45high cells, I) T Cell frequency (CD19-, CD3e+), J) B Cell frequency (CD19+, CD3e-) (n=6-8/group). Each point represents data from an individual mouse. White data points represent WT mice and grey data points represent ASO mice. Data is combined from three independent experiments. Data analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001



Supplement Figure 7: Immune cell characterization in small intestine, large intestine, and spleen of PLX5622-treated mice

Supplement Figure 7K-O: Spleen levels of K) CD45, CSF1r+ high cells L) CD45+, CSF1r low cells, M) CD11b+, CD45high cells, N) T Cell frequency (CD19-, CD3e+), O) B Cell frequency (CD19+, CD3e-) (n=3-6/group). Each point represents data from an individual mouse. White data points represent WT mice and grey data points represent ASO mice. Data is combined from three independent experiments. Data analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001

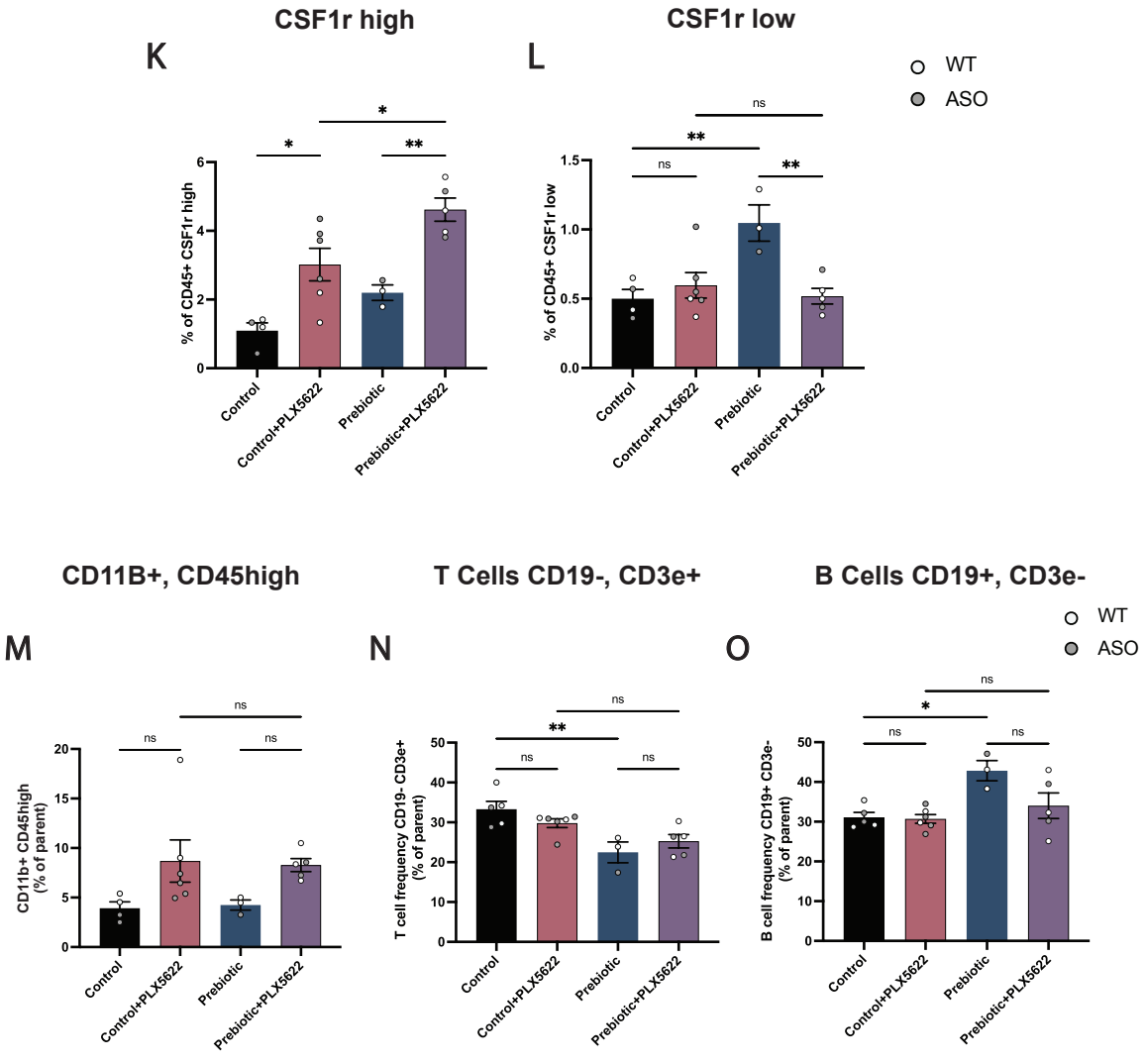


Table 1-Composition of custom-made prebiotic diets

Ingredient	Control diet TD. 170167 (g/Kg)	Prebiotic #1 diet TD.170864 (g/Kg)	Prebiotic #2 diet TD.170865 (g/Kg)
Casein	200	200	200
L-Cystine	3	3	3
Corn Starch	392.234	242.234	242.234
Maltodextrin	132	82	82
Sucrose	150	150	150
Soybean oil	70	70	70
Mineral mix AIN-93G-MX (94046)	35	35	35
Vitamin Mix, AIN-93-VX (94047)	15	15	15
Choline Bitartrate	2.75	2.75	2.75
Vitamin K1, phylloquinone	0.002	0.002	0.002
TBHQ, antioxidant	0.014	0.014	0.014
Pectin (from apple, Best Botanicals)	–	–	–
Resistant Starch (from potato, Bob's Red Mill)	–	–	100
Chitin-Glucan (KitoZyme)	–	–	100
Wheat Bran (Bob's Red Mill)	–	100	–
Resistant Maltodextrin (Nutriose, Roquette)	–	100	–
Fructooligosaccharides - FOS (Nutraflora, Ingredion)	–	–	–

Materials and Methods

Animals

Breeding: The Thy1-α-synuclein (ASO; line 61) mouse line was used for all experiments in this study (Chesselet et al., 2012; Rockenstein et al., 2002). Male BDF1 mice were crossed with female ASO mice expressing the α-synuclein transgene on the X chromosome to generate WT and ASO littermates. Mice were weaned at p21 and housed by genotype on the day of weaning. Male mice were used for all experiments in this study.

Diet experiments: Mice were switched from standard chow to either the cellulose-free control diet or high-fiber prebiotic diet at 5-6 weeks of age and housed in sterile, autoclaved cages with sterile water. Custom fiber mixes were sent from Purdue University for formulation at Envigo Teklad (Madison, WI, USA).

PLX5622 was acquired from DC Chemicals and incorporated in the cellulose-free and prebiotic diets at a dosage of 1,200 ppm. Mice were switched to the PLX5622 diet at 5-6 weeks of age. Diets were replenished weekly and food intake was measured weekly. Mice were monitored by the lead investigator and Caltech veterinary staff for adverse health effects.

All animal experiments were done under the guidance and approval of Caltech's Institutional Animal Care and Use Committee (IACUC).

Motor Testing

A full battery of motor tests was performed at 22 weeks of age. All motor testing was completed in the same room in a biological safety cabinet between the hours of 6 and 10 of the light phase. Motor testing was completed as described in (Fleming et al., 2004; Sampson et al., 2016). Motor tests were done in the following order: Day 1: beam traversal training, pole training; Day 2: beam traversal training, pole training, wire hang; Day 3: beam traversal test, pole test, hindlimb score, adhesive removal; Day 4: fecal output. Mouse cages were not changed during the duration of testing.

Beam traversal: Time to cross, errors per step, and number of steps were tested using a plexiglass beam 1 m in length. The beam was constructed of four individual segments, with decreasing width of 1 cm increments along the length of the beam (3.5 cm, 2.5 cm, 1.5 cm, and 0.5 cm). Mice were trained for two consecutive days prior to testing on day 3. On each training day, mice were prompted to cross the beam for three consecutive trials. On testing day mice were recorded using a GoPro camera for analysis of errors per step and number of steps.

Pole descent: Time to descend a 24-inch pole wrapped in mesh liner was recorded. The pole was placed in the animal's home cage and mice were trained for two consecutive days prior to testing on day 3. Three trials were performed on day 1 of training: trial 1: mice were gently placed head down on the pole 1/3 of the distance from the base, trial 2: mice were placed head down on the pole 2/3 of the distance from the base, trial 3: mice were placed head down on the top of the pole. On day 2 of training, mice were placed on

the top of the pole for three consecutive trials. On testing day, mice were placed on the top of the pole for three trials of testing. The timer was stopped once one of the front hindlimbs touched the base of the pole. Time to descend was averaged across all three trials.

Adhesive removal: A 0.25 in. adhesive, round sticker was placed on the nose of the mouse. The mouse was subsequently placed in its home cage (without cagemates) and time to remove the adhesive was recorded. Time to remove was averaged across two trials.

Wire hang: Mice were placed in the middle of a rectangular wire grid placed over a sterile, clean cage. the wire grid was gently inverted with the mouse hanging over the cage. Time to fall was recorded as the time between grid inversion and the mouse falling off the grid. Maximum time was set to 60 seconds. Time to fall was averaged across two trials.

Hindlimb score: Mice were gently held upwards in the air by the mid-section of their tail and hindlimb movement was observed. Mice were given a score of 0, 1, 2, or 3 depending on the movement and flexibility of their rear hindlimbs. The score was assessed by two experimenters and the average score was reported.

Scores were assigned as follows:

0: rear hindlimbs were flexible and mobile, with a complete range of motion; no inward clasping was observed.

1: rear hindlimbs exhibited mild rigidity with hindlimbs orienting inward slightly.

2: rear hindlimbs oriented inward, but were not completely clasped.

3: rear hindlimbs were firmly clasped together.

Microglia Isolation and Sequencing

Microglia isolation: Microglia were isolated from mouse brains at 22 weeks of age. For all experiments, samples were pooled from 4-6 mice/treatment group. Mice were anesthetized and perfused with ice-cold PBS. Brain regions of interest were dissected and homogenized using mechanical dissociation. Single cell suspensions were obtained using a Dounce homogenizer. A 37/70 Percoll density gradient was used to separate cells from debris and myelin. Following Percoll separation, cells were washed and stained with Cd11B (1:1,000, Biolegend), CX3CR1 (1:10,000, Biolegend), CD45 (1:1,000, Biolegend), and DAPI (1:10,000, Sigma-Aldrich). All steps were performed in microglia staining buffer (1X HBSS, 1% BSA and 1 mM EDTA). Cells were sorted in a FACSAria III Fusion flow cytometer (BD Biosciences). Live CD11b+, CX3CR1+, and CD45 (low) cells were identified as microglia and collected for downstream analysis. The full protocol can be found at protocols.io (https://doi.org/10.17504/protocols.io.kqdg3p7bel25/v1)

Single cell sequencing: The v3.1 Chromium Next GEM single cell reagent kit from 10x genomics was used for scRNAseq of FACS-purified microglia. Between 2-4,000 cells were loaded on the Next GEM chip for substantia nigra samples, with 1,000-1,700

cells/group recovered for analysis. For striatum samples, approximately 8-16,000 cells were loaded on the Next GEM chip, with 5-10,000 cells/group recovered for analysis. Library construction was completed according to the manufacturer's instructions. Samples were tagged with a unique sample index, pooled, and sequenced with an average depth of 111k reads/cell on a NovaSeq 6000 sequencing platform (Illumina). Cell Ranger software (10X Genomics) was used for sequence alignment, cluster analysis, and identification of differentially expressed genes between groups. ShinyGO was used for gene ontology and pathway analysis (Ge, Jung, and Yao, 2020).

Single-cell transcriptomic analysis: The data were first filtered by removing cells with less than 200 genes and genes that were expressed in less than 100 cells. Gene counts were normalized by dividing the number of times a particular gene appeared in a cell (gene cell count) by the total gene counts in that cell. Counts were multiplied by a constant factor (5,000), a constant value of 1 was added to avoid zeros, and then the data were log transformed. Data analysis steps including Leiden clustering, differential gene expression analysis, and plotting of marker genes were performed using the Scanpy package (Wolf, Angerer, and Theis 2018).

ATAC Seq: FACS-purified microglia were collected as described above and resuspended in 50 µL of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% IGEPAL CA-630). Cells were spun down at 500 xg for 10 min at 4ºC. Supernatant was discarded and a transposition reaction was performed on the cell pellet using the Illumina Tagment DNA enzyme and buffer kit. Samples were purified using the Zymo

ChIP DNA clean and concentrator kit and transposed DNA was eluted in elution buffer. Two independent trials were completed for the experiment.

Immunohistochemistry:

Sectioning: 22-week-old WT and ASO mice were anesthetized with pentobarbital (Euthasol). Mice were perfused with ice-cold phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA). Brains were removed and placed in tissue culture plates with 4% PFA for 48 hours before transfer to PBS+ 0.05% sodium azide. Whole brains were embedded in agarose and sliced coronally into 50 μM sections using a vibratome. Free-floating sections were placed in PBS+ 0.05% sodium azide and stored at 4ºC until staining.

Staining: Sections were permeabilized for 30 minutes in 3% BSA, 0.5% Triton X-100 in PBS, blocked for 1 hour in 3% BSA, 0.1% Triton X-100 in PBS, and stained with IBA-1 (1:1,000, Wako, anti-rabbit) and tyrosine hydroxylase (Th) (1:1,000, Abcam, anti-chicken) overnight at 4ºC (protocol adapted from Datta et al., 2018). Sections were then stained with anti-rabbit IgG AF-647 (1:1,000, Life Technologies) and anti-chicken IgG AF-594 (1:600, Jackson ImmunoResearch). Slices containing brain regions of interest were mounted on a cover slip using ProLong Diamond anti fade mountant with 4',6-diamidino-2-phenylindole (DAPI). Coverslips were stored at 4ºC until imaging.

Imaging: Images were obtained on a Zeiss LSM800. For diameter measurements: images were taken with a 20X objective, with 3 pictures taken per brain region of interest. Imaris

Software was used to measure the diameters of cells, with 30-70 cells counted per brain region/animal. For 3D reconstruction: z-stack images were taken with 1.00 µm steps in the z-direction with a 40X objective. 3D reconstruction was done in the Imaris Software, with 3-6 cells analyzed per brain region/animal.

α-synuclein Aggregation Assays:

Substantia nigra and striatum were dissected on ice from 22-week-old mice and stored at -80ºC until used.

Protein extraction: Brain tissues were lysed using Tissue Extraction Reagent (ThermoFisher) and protease inhibitor. Samples were homogenized for 90 seconds using a bead beater and were placed directly on ice for 10-15 minutes following homogenization. Lysates were centrifuged at 10k rpm for 5 minutes and supernatants were collected and stored at -80ºC for later use. The full protocol can be found at protocols.io (https://doi.org/10.17504/protocols.io.5jyl896o6v2w/v1)

α -synuclein aggregation: Levels of aggregated α-synuclein were determined using the dot blot assay. Samples were quantified using the Pierce BCA Protein assay kit (Thermo Fisher) and normalized to equal concentrations between 0.5-1.0 ng/µL in water. 1 µg of sample was spotted on dry nitrocellulose membrane (0.45 µm). Samples were blocked in 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) and stained with anti-aggregated α-synuclein antibody (1:1,000, Abcam) overnight at 4ºC. The next day, blots were stained with anti-rabbit IgG-HRP (1:1,000, Cell Signaling) for 2 hours. Signal was detected using Clarity chemiluminescence substrate (Bio-Rad) and imaged on a Bio-

Rad digital imager. Integrated density is reported as the intensity of an identically-sized area of each dot for each sample. The full protocol can be found at protocols.io (https://doi.org/10.17504/protocols.io.261gen2xdg47/v1)

RNA Extraction and qPCR:

Brain regions were dissected on ice from 22-week-old mice and stored at -80ºC in RNAlater solution (Thermo Fisher) until RNA extraction.

RNA extraction: RNA was extracted using either Direct-zol RNA Microprep or Miniprep kit (Zymo Research) depending on the size of the brain region.

qPCR: RNA was transcribed using the iScript ™ cDNA synthesis kit (Bio-Rad) per the manufacturer's instructions. SYBR Green master mix was used for qPCR reactions. Primers used for experiments were: HDAC1: 5'-GAACTGCTAAAGTACCACC-3' & 5'-CATGACCCGGTCTGTAGTAT-3; HDAC2: 5'-CGGTGTTTGATGGACTCTTTG-3' & 5'-CCTGATGCTTCTGACTTCTTG-3'; HDAC6: 5'-CTGCATGGCATCGCTGGTA-3' & 5'-GCATCAAAGCCAGTGAGATC-3' ; HDAC7: 5'-CTCGGCTGAGGACCTAGAGA-3' & 5'-CAGAGAAATGGAGCCTCTGC-3' ; HDAC9: 5'-GCGGTCCAGGTTAAAACAGAA-3' & 5'-GCCACCTCAAACACTCGCTT-3' ; GAPDH: 5'-CATGGCCTTCCGTGTTCCTA-3' & 5'- CCTGCTTCACCACCTTCTTGAT-3'; FFAR2: 5'-TTCCCATGGCAGTCACCATC-3' & 5'-TGTAGGGTCCAAAGCACACC-3'; FFAR3: 5'-ACCGCCGTCAGGAAGAGGGAG-3' & 5'TCCTGCCGTTTCGCSTGGTGG-3'

Isolation of Immune Cells from Intestinal Lamina Propria/Spleen and Flow Cytometry:

For isolation of intestinal lamina propria cells, the small and large intestines were dissected and placed immediately into ice-cold PBS. After mesenteric fat and Peyer's patches (small intestine) were removed, the intestines were longitudinally opened and luminal contents were washed out with cold PBS. Tissue pieces were washed for 10 min in 1 mM dithiothreitol (DTT)/PBS at room temperature on a rocker to remove mucus, followed by a wash for 25 min in 10 mM EDTA/30 mM HEPES/PBS at 37ºC on a platform shaker (180 rpm) to remove epithelium. After a 2 min wash in complete RPMI, tissue was digested in a 6-well plate for 1.5hrs in complete RPMI with 150 U/mL (small intestine) or 300 U/mL (large intestine) collagenase VIII (Sigma-Aldrich) and 150 µg/mL DNase (Sigma-Aldrich) in a cell culture incubator (5% CO_2). Tissue digests were passed through a 100 µm cell strainer and separated by centrifugation (1,200 xg for 20 min) using a 40/80% Percoll gradient. Immune cells were collected at the 40/80% interface. For the spleen, the tissue was passed through a 100 µm cell strainer and incubated in red cell lysis buffer (Sigma-Aldrich) for 8 min at room temperature. Both spleen and intestine immune cells were washed with 0.5% BSA/PBS before staining and fixation (eBioscience Foxp3 / Transcription Factor Staining Buffer Set).

For flow cytometry staining, CD16/32 antibody (eBioscience) was used to block non-specific binding to Fc receptors before surface staining. Immune cells were stained with antibodies against the following markers: CD103 (PerCP-efluor710), CD11b (SuperBright645), CD11c (FITC), CD19 (FITC), CD3e (PE), CD4 (APC), CD45.2

(BV421), CD64 (APC-Cy7), CD8a (APC-e780), CSF1R (PE), Ly6C (APC), MHCII I-A/I-E (PE or PerCP-efluor710), TCRβ (PerCP-Cy5.5). For some panels, a lineage marker mix (Lin) contained TCRβ, B220, Ly6G and Siglec-F (PE-Cy7). Live and dead cells were discriminated by Live/Dead Fixable Aqua Dead Cell Stain Kit (Invitrogen).

Gut Microbiome Profiling:

Metagenomic Sequencing: Shotgun sequencing libraries were generated using the Kapa HyperPlus protocol on gDNA extracted from mouse fecal pellets. Samples were sequenced using 150 bp paired end reads on an Illumina NovaSeq 6000 at the UCSD IGM Genomics Center.

Metagenomic Analyses: Quality control filtering and read alignment of metagenomic reads was conducted with Qiita (study-id 13244). First, adapter removal and quality trimming were conducted using Atropos v1.1.24. To generate taxonomic and functional gene-level profiles, we applied the Woltka v0.1.1 pipeline to align reads against the Web of Life database (Zhu et al., 2019) using Bowtie2 v2.3.0(Langmead and Salzberg, 2012), followed by generation of Operational Genomic Units (Zhu et al., 2021). Downstream statistical analyses and data visualization was conducted in R (v4.1.0). For community-level measures, including alpha- and beta-diversity, Woltka-generated taxonomic predictions at the species level were rarefied to an even depth of 321,980 counts. Alpha-diversity metrics including Observed Species, Simpson's Evenness, and Gini's Dominance were calculated using the microbiome R package and tested for statistical significance using a one-way ANOVA for treatment group and post-hoc Tukey's test for

pairwise comparisons. Assessment of between-sample diversity was accomplished using the Bray-Curtis distance. We estimated metadata-explained variance using the Bray-Curtis distance with permutational multivariate analysis of variance (PERMANOVA) with 9,999 permutations followed by multiple hypothesis testing corrections using the Benjamini-Hochberg method (FDR = 0.1). Differential abundance testing was conducted using Multivariable Association with Linear Models (MaAsLin2) (Mallick et al., 2021). For data preparation, we applied a 10% prevalence filter, total sum scale normalization, and an arcsine square root transformation for variance stability. We then applied a feature-level-specific variance filter based on the variance distribution and the number of features present at each level. MaAsLin2 linear models were fit with genotype and diet variables as fixed effects.

SCFA fecal measurements (LC-MS): Fecal samples were collected from mice at 22 weeks of age and stored at -80 ºC until analysis. Sample preparation: Mouse fecal samples were extracted and derivatized as described previously (Chan et al., 2017). Briefly, ice-cold extraction solvent (1:1 v/v acetonitrile/water) was added to fecal sample at a ratio of 2 µL:1 mg sample and internal standard mix to a final concentration of 100 µM. The suspension vortex mixed for 3 min at room temperature, sonicated for 15 min, and then centrifuged at 18,000 x g for 15 min at 4 ºC. An aliquot of 100 µL was subsequently derivatized using a final concentration of 10 mM aniline and 5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (ThermoFisher) for 2 h at 4 ºC. The derivatization reaction was quenched using a final concentration of 18 mM succinic acid and 4.6 mM 2-mercaptoethanol for 2 h at 4 ºC. All samples were stored at 4

°C until analysis on the same day. Mixed calibrators of acetic acid, propanoic acid, butyric acid and isobutyric acid (10nM - 10×10^3 nM) (Sigma-Aldrich) together with single- and double- blanks, spiked with internal standard mix (Acetic acid-d3, propanoic acid-d2, butyric acid-d2) (Pointe-Claire) to a final concentration of 100 µM were prepared and subjected to the same sample preparation procedure as fecal samples. The full protocol can be found at protocols.io (https://doi.org/10.17504/protocols.io.bp2l61rrkvqe/v1)

Liquid Chromatography Mass Spectrometry (LC-MS): Derivatized samples were analyzed using an ultra-high-performance liquid chromatography (UHPLC) system 1290 connected to a quadrupole time of flight (Q-TOF 6545) mass spectrometer (Agilent Technologies) equipped with an orthogonal DUAL AJS-ESI interface. Samples were subjected to reverse phase C18 separation (Phenomenex Scherzo SS-C18 100 x 2 mm) and data were collected in positive ion mode. Data were acquired from 50 to 750 m/z-1 at 2 spectra s^{-1}. Electrospray ionization (ESI) source conditions were set as follows: gas temperature 325 °C, drying gas 9 L min^{-1}, nebulizer 35 psi, fragmentor 125 V, sheath gas temperature 350 °C, sheath gas flow 8 L min^{-1}, nozzle voltage 1000 V. For reverse phase C18 chromatographic separation, a two-solvent gradient running at 0.3 mL min^{-1} (Mobile Phase: A: 100:0.1 Water:Formic Acid, B: 100:0.1 Isopropanol:Formic Acid) was used. the column was equilibrated at 15% B for 1 min and a sample was introduced. The solvent ratio was then increased from 15% B to 100% B over 13 min and then reduced back to 15% B over 2 min. Injection volume was 5 µL with a column temperature of 45 °C. The LC-MS/MS data acquired using Agilent Mass Hunter Workstation (.d files) were

processed in quantitative analysis software (Agilent Technologies) for quantitative analysis of samples. The linear calibration plots for acetic acid, propanoic acid, butyric acid and isobutyric acid were constructed using peak area ratios of each analyte to the IS versus the concentrations of calibrators (x) with 1/x weighting, and the least squares linear regression equations were obtained as the calibration equations for individual analytes.

SCFA brain measurements (UHP-LC): Striatum and substantia nigra were dissected from 22-week-old mice, placed in dry ice, and stored at -80ºC until analysis. Samples were analyzed by BIOTOOLS CO. using an ultra-high-performance liquid chromatography (UHPLC) system. Brain tissue samples were extracted with 70% methanol for 30 mins, using a sample:solvent ratio of 1 mg:40 µL. The sample was centrifuged at 21,380 rcf for 5 min at 4°C. The supernatant was used for derivatization procedures. Each sample was mixed with 5 µL of 0.1 mM internal standard and 200 µL each of pyridine, 1-EDC-HCl, and 2-NPH-HCl solutions as reaction- assistive agents, and reacted at 45°C for 20 min. 100 µL of potassium hydroxide solution was added (to stop the reaction) and reacted at 45°C for 15 min. After cooling, the mixture was ultrasonicated with 1 mL of phosphoric acid aqueous solution and 2 mL of ether for 3 min and then centrifuged for 5 min at 2,054 rcf. The ether layer was collected and spun-dry. The sample was reconstituted with 25 µl MeOH. Mass analysis: Each sample (2 µL) was injected into a vanquish ultra-high-performance liquid chromatography (UHPLC) system coupled with SCIEX QTrap® 5500. UHPLC parameters were set as follows: A CSH 1.7 µm, 2.1x100 mm column

(Waters) was used. The column oven temperature was set at 45°C. The binary mobile phase included deionized water containing 5 mM ammonium acetate as solvent A, and acetonitrile with 5 mM ammonium acetate as solvent B. The flow rate was 0.35 mL/min with a linear gradient elution over 15 min. Reagent 1: Pyridine (Sigma-Aldrich) was adjusted with methanol to 3% (V/V) (Weng et al., 2020).

Statistical Analysis:

Graphpad Prism software (version 9.0) was used for statistical analysis. Data presented represent mean ± SEM, with each data point representing values from an individual mouse. All behavioral and molecular data were analyzed by two-way ANOVA followed by Tukey's multiple comparisons test, unless stated otherwise. *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$

Data Availability

All datasets generated or analyzed in this study can be found through the Zenodo depository: https://doi.org/10.5281/zenodo.6377704 All experimental protocols can be found on protocols.io.

List of Reagents

Reagent type (species) or resource	Designation	Source of reference	Identifier	Addition-al informat-ion
Mus musculus	Thy1-α-synuclein (line 61)	(Chesselet et al., 2012; Rockenstein et al., 2002)	ASO	
Antibody	Anti-beta actin, rabbit polyclonal	Abcam	Ab8227	1:1,000
Antibody	Anti-aggregated α-synuclein, rabbit polyclonal	Abcam	Ab209538	1:1,000
Antibody	Anti-Iba1, rabbit polyclonal	Wako	019–19741	1:1,000
Antibody	Anti-tyrosine hydroxylase, chicken polyclonal	Abcam	ab76442	1:1,000
Antibody	Anti-rabbit IgG-647, donkey polyclonal	Life Technologies	1874788	1:1,000
Antibody	Anti-chicken IgG-594, donkey polyclonal	Jackson Immunoresearch	703-585-155	1:600
Antibody	Anti-rabbit IgG, HRP-linked	Cell Signaling	7074	1:1,000
Antibody	Anti-mouse/human CD11b-APC, rat monoclonal	BioLegend	101211	1:1,000
Antibody	Anti-mouse CX3CR1-PE/Cyanine7, mouse monoclonal	BioLegend	149016	1:10,000
Antibody	Anti-mouse CD45-Alexa Flour 488, rat monoclonal	BioLegend	103121	1:1,000
Antibody	DAPI	Sigma-Aldrich	10236276001	1:10,000
Antibody	Aqua Viability Dye	ThermoFisher/Invitrogen	L34957	1:1,000
Antibody	CD16/CD32 Monoclonal Antibody (93), eBioscience™ (1mg)	ThermoFisher	14-0161-86	1:100
Antibody	CD3e Monoclonal Antibody (145-2C11), PE, eBioscience™	ThermoFisher	12-0031-82	1:200

Antibody	CD4 Monoclonal Antibody (GK1.5), APC, eBioscience™	ThermoFisher	17-0041-83	1:200
Antibody	TCR beta Monoclonal Antibody (H57-597), PerCP-Cyanine5.5, eBioscience™	ThermoFisher	45-5961-82	1:200
Antibody	CD8a Monoclonal Antibody (53-6.7), APC-eFluor 780, eBioscience™	ThermoFisher	47-0081-82	1:200
Antibody	CD11c Monoclonal Antibody (N418), FITC, eBioscience™	ThermoFisher	11-0114-82	1:200
Antibody	CD170 (Siglec F) Monoclonal Antibody (1RNM44N), PE-Cyanine7, eBioscience™	ThermoFisher	25-1702-82	1:200
Antibody	Ly-6C Monoclonal Antibody (HK1.4), APC, eBioscience™	ThermoFisher	17-5932-82	1:200
Antibody	CD103 (Integrin alpha E) Monoclonal Antibody (2E7), PerCP-eFluor 710, eBioscience	ThermoFisher	46-1031-82	1:200
Antibody	CD64 Monoclonal Antibody (X54-5/7.1), APC-eFluor 780, eBioscience™	ThermoFisher	47-0641-82	1:200
Antibody	CD11b Monoclonal Antibody (M1/70), Super Bright 645, eBioscience™	BioLegend	64-0112-82	1:200
Antibody	BV421 anti-mouse CD45.2	Tonbo	109831	1:200
Antibody	PE-Cy7 anti-mouse Ly6G	Tonbo	60-1276-U100	1:200
Antibody	PE-Cy7 anti-mouse TCRb	Tonbo	60-5961-U100	1:200
Antibody	PE-Cy7 anti-mouse B220	Tonbo	60-0452-U100	1:200
Antibody	FITC anti-mouse CD19	Tonbo	35-0193-U500	1:200
Antibody	PE Anti-Mouse MHC Class II (I-A/I-E) (M5/114.15.2)	Tonbo	50-5321-U100	1:200
Antibody	PE anti-mouse CD115 (CSF-1R) Antibody	BioLegend	135506	1:200
Antibody	MHC Class II (I-A/I-E) Monoclonal Antibody (M5/114.15.2), PerCP-eFluor 710, eBioscience™	ThermoFisher	46-5321-82	1:200
Reagent	eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set	ThermoFisher	00-5523-00	

Reagent	PLX5622	DC Chemicals	DC21518	
Reagent	IL-6 Mouse ELISA kit	ThermoFisher	88-7064-88	
Reagent	TNF-α Mouse ELISA Kit	ThermoFisher	88-7324-77	
Reagent	Tagment DNA enzyme and buffer kit	Illumina	20034197	
Reagent	Prolong Diamond antifade mountant with DAPI	Invitrogen	P36971	
Reagent	Tissue Extraction Reagent I	ThermoFisher	FNN0071	
Reagent	Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1	10x Genomics	1000128	
Reagent	Chromium Next GEM Chip G Single Cell Kit	10x Genomics	1000127	
Reagent	Single Index Kit T Set A	10x Genomics	2000240	
Reagent	ChiP DNA clean and concentrator	Zymo	D5205	
Reagent	Direct-zol RNA Microprep	Zymo	R2062	
Reagent	Direct-zol RNA Miniprep	Zymo	R2050	
Reagent	iScript ™ cDNA synthesis kit	Bio-Rad	1708890	
Reagent	Clarity ™ Western ECL Substrate	Bio-Rad	1705060	
Sequence-based reagent	HDAC1: 5'-GAACTGCTAAAGTACCACC-3' 5'-CATGACCCGGTCTGTAGTAT-3'			
Sequence-based reagent	HDAC2: 5'-CGGTGTTTGATGGACTCTTTG-3' 5'-CCTGATGCTTCTGACTTCTTG-3'			

Sequence-based reagent	HDAC6: 5'-CTGCATGGCATCGCTGGTA-3' 5'-GCATCAAAGCCAGTGAGATC-3'			
Sequence-based reagent	HDAC7: 5'-CTCGGCTGAGGACCTAGAGA-3' 5'-CAGAGAAATGGAGCCTCTGC-3'			
Sequence-based reagent	HDAC9: 5'-GCGGTCCAGGTTAAAACAGAA-3' 5'-GCCACCTCAAACACTCGCTT-3'			
Sequence-based reagent	GAPDH: 5'-CATGGCCTTCCGTGTTCCTA-3' 5'-CCTGCTTCACCACCTTCTTGAT-3'			

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Chapter 4

CONCLUSION

Microbiome research in the past and present

Our understanding of how the gut microbiome shapes host health and influences disease is constantly evolving as new research findings emerge. A critical takeaway from the research of the past few decades is the CNS, although shielded partially by the BBB, is shaped by a multitude of factors in the periphery, including the gut microbiome. Understanding how communication along the gut-brain axis influences the host during homeostatic and disease states is a central research theme among a growing number of research labs across the globe.

The studies that emerged in the past ~10 years characterized differences between GF and SPF mice, noting variations in CNS development and behavior (Luczynski et al., 2016). Studies in the present day are more nuanced, attempting to characterize pathways of communication between the gut and brain and identify the specific pathways and molecule(s) mediating these interactions. With a stronger mechanistic insight into gut-host interactions, researchers can begin to ask the critical question of how can we change the microbiome to confer protective disease outcomes. Gaining a better understanding of gut-host interactions is critical given emergent disease trends. Incidence rates of metabolic, immune, neuropsychiatric, and neurodegenerative diseases are on the rise and are projected to continue to increase over the next few decades. Interestingly, preclinical and clinical studies have illustrated a role for the gut microbiome in many of these conditions.

Microbiome as a tool to treat disease

The work presented in Chapter 3, as well as other recently published research, reveal that changes to the gut microbiome composition in various preclinical paradigms influence the course of disease. These findings have broad implications for the medical and research community. As discussed earlier in this thesis, gut microbiome therapies encompass several modalities (prebiotics, probiotics, FMT) (Sorbara and Pamer, 2022). While the presence of gut-targeted therapies in the clinic is currently limited, advancements in preclinical studies and ongoing clinical trials may expedite their widespread application.

Developments in gut microbiome research may transform the state of diagnostics and therapeutics in the next few years. This holds especially true for several neurodegenerative and neuropsychiatric conditions as traditional pharmacological interventions have proven ineffective. Utilizing gut-based therapeutics in the prodromal phase of PD may be especially beneficial. Typically, classic motor symptoms of PD develop only when an individual has lost ~80% of their dopaminergic neurons, rendering current therapies unsuccessful in reversing the disease and underscoring the need for alternative treatments (Cheng, Ulane, and Burke, 2010). The current gold-standard treatment for PD is Levodopa (L-Dopa), a dopamine precursor converted to dopamine upon entry in the brain. While L-Dopa has proven effective in improving resting tremor, muscle rigidity and bradykinesia, treatment has many undesirable side effects, and efficacy begins to wane after a few years (Poewe et al., 2010). Since GI complications typically precede the onset of motor and cognitive symptoms in PD, the microbiome

offers an opportunity for early-stage intervention for patients, leading to improved quality of life and prognosis.

Gut-targeted therapies may also have huge benefits for individuals suffering from neuropsychiatric conditions. The CDC estimates that 1 in 5 Americans has a mental illness in a given year (CDC.org). The COVID-19 pandemic has shed light on the pervasiveness of mental illness in our society, while also exacerbating feelings of stress, anxiety, and loneliness. The co-occurrence rate of GI conditions and neuropsychiatric conditions such as depression and anxiety is high, and several studies to date have reported differences in microbiome makeup in individuals with and without major depressive disorder (MDD) (Cheung et al., 2019; Söderquist et al., 2020). Current standard of care for MDD patients are SSRIs and other antidepressants, which often accompany undesirable side effects and have a fairly low response rate (40-60%) (AHRQ.org). Clinical trials to explore the safety and efficacy of FMT and probiotics for individuals with MDD are currently underway.

Microbiome in the future

The development of innovative microbiome technologies in parallel to ongoing preclinical work has the potential to radically transform the state of gut microbiome research. Leveraging advances in precision medicine, artificial intelligence, and machine learning technology may allow the gut microbiome to be used not only as a therapeutic target, but as a biomarker for disease and/or a diagnostic tool.

The application of precision medicine in gut microbiome research encompasses two main platforms: 1) developing custom probiotics and dietary treatment plans to alter the gut microbiome for a desired health outcome and 2) using an individual's gut microbiome fingerprint to inform drug prescription, dosage, and tolerability and predict treatment response. To date, there are several companies (Viome, Zoe, Ombre) offering at-home gut microbiome tests to develop personalized probiotics and nutrition plans tailored to individual users. The microbiome can also be used as a tool in the treatment and diagnosis of disease. Studies have shown that gut microbiome composition can predict response to cancer immunotherapy treatment (Gopalakrishnan et al., 2018), blood glucose spikes in response to diet (Zeevi et al., 2015), and efficacy of anti-depressants (Bharwani et al., 2020). Going one step further, integrating machine learning in precision medicine studies has powerful implications. Using gut microbial signatures along with other relevant input parameters, researchers can train algorithms to identify biomarkers of disease and make other predictions related to patient diagnosis and prognosis (Rajkomar, Dean, and Kohane, 2019) .

Limitations and challenges

There are several research and technical limitations that must be addressed before microbiome diagnostics and therapeutics can take strong foothold. First, the research community must come to a consensus on the definition of a "healthy microbiome." This is difficult to achieve given that interindividual variability in microbiome composition is high (Falony et al., 2016; Gilbert et al., 2018). Two individuals who appear otherwise "healthy" may have vastly different microbiome compositions. While establishing

healthy baselines is attainable for less complex measures of health such as weight, body temperature, or blood glucose levels, developing a microbiome profile that represents the pinnacle of health is a challenging feat. Moreover, the human microbiome is influenced by a variety of factors and therefore the model of a healthy microbiome may need to be adapted depending on a person's geography, diet, medication use, and diagnosed conditions (Falony et al., 2016).Variations in sample collection and data analysis pipelines between research studies adds another element of complexity in human microbiome studies.

Another limitation to be addressed is the type of observational study used in microbiome studies. To date, advances in sequencing and -omics technology have accelerated publication of microbiome-wide association studies (MWAS) that attempt to link genetic and molecular features of the microbiome to disease (Gilbert et al., 2016). However, these association studies are most often cross-sectional, sampling the microbiome in different populations at a single point in time (Sinha et al., 2018). While these studies are useful in identifying broad differences between experimental groups, their results can be difficult to interpret as an individual's microbiome is dynamic and variable over the short term (Gilbert et al., 2018). Therefore, repeating the experiment at different times may yield different results. Conversely, longitudinal studies allow researchers to sample an individual's microbiome over time. These studies allow scientists and clinicians to establish a person's healthy baseline microbiome profile and utilize temporal shifts in the microbiome to gain insight into an individual's health.

Lastly there remain many unknowns in our understanding of the gut microbiome. Most preclinical and sequencing studies to date focus on the bacteria that reside in the GI tract, since they make up the vast majority of the gut microbiome. However, there is a world beyond bacteria that includes the mycobiome (fungi), virome (virus), and archaeome (archaea) (Matijašić et al., 2020). Integrating information from non-bacterial commensals in our gut is crucial for our understanding of gut health and human disease.

The rapid advancements of new tools, analysis pipelines and collaborations makes researchers in the field equipped to face these challenges. The next 10-20 years of microbiome research has the potential to transform how we think of human health and revolutionize the drug and therapeutic development landscape.

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