Targeting pathological changes in the gut protects the CNS from neuropathology.

Benjamin Tyler Charpentier

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TARGETING PATHOLOGICAL CHANGES IN THE GUT PROTECTS THE CNS FROM NEUROPATHOLOGY

By

Benjamin Tyler Charpentier
B.S. Bellarmine University, 2016
M.A. University of Louisville, 2019

A Dissertation Submitted
to the faculty of the
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Department of Anatomical Sciences and Neurobiology
University of Louisville
Louisville, Kentucky

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A Dissertation Approved on

July 29, 2022

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DEDICATION

This dissertation is dedicated to my beloved wife for her continuous love and support and to my son and future children, that I may provide, protect, and teach you.
ACKNOWLEDGMENTS

Firstly, I would like to thank Dr. Whittemore and Dr. Barve for their mentorship and guidance throughout my studies and research. Both Dr. Whittemore and Dr. Barve had significant impact to progress my personal growth and development under their leadership. These contributions cannot be overstated, and I am incredibly grateful to have done my studies at the University of Louisville and work under their guidance. Thank you, Dr. Myers, for your training in immunohistochemical method and analysis. I would also like thank for Dr. Chilton and Dr. Yaddanapudi for their training in immune cell isolation, staining, and flow cytometry as well as Dr. Tyagi and her lab’s collaboration in behavioral studies in chapter 3 as well as Balor college of medicine and their collaboration with sequencing and microbiome analysis. I want to personally thank all the members of the Whittemore and Barve labs for aiding in experiments, data analysis, and friendships. I am grateful to my committee members Dr. McClain, Dr. McGee, and Dr. Stirling as they have provided me with their expertise, constructive comments, and their time.

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ABSTRACT

TARGETING PATHOLOGICAL CHANGES IN THE GUT PROTECTS THE CNS FROM NEUROPATHOLOGY

Benjamin Charpentier

July 29, 2022

There is growing evidence that the health of the gut microbiome (GM) has an important role on multiple host organ systems. Ethanol-related neuropathologies and Alzheimer’s disease (AD) currently have no effective clinical treatment. Therefore, we developed a novel mouse model of fecal matter transplantation (FMT) utilizing the microbiome from alcoholic hepatitis (AH) patients, characterized by a loss of butyrogenic potential. Minimal neuroinflammation occurred in AH-FMT mice, but significant increases in the endoplasmic reticulum stress response (ERSR) across the majority of neurons in the prefrontal cortex and hippocampus were observed. In the cerebellum, Purkinje cells were specifically affected. Targeting the loss of butyrate reduced the ERSR as well as low-grade inflammation.

The AD 3xTg mouse model was assessed for the development of dysbiosis. At 12 months these mice developed dysbiosis, characterized by the loss of butyrate-producing bacteria. Butyrate supplementation did positively impact inflammation, oxidative stress, and neurofibrillary tangle formation. Functionally, cognitive decline was ameliorated in butyrate-supplemented mice.

Aldose reductase (AR) is the rate-limiting enzyme in the polyol pathway. Biproducts of this pathway yield increases in inflammatory products and decreases in antioxidants. Lipopolysaccharide (LPS) was injected intraperitoneally in mice to mimic
endotoxemia. The brains and livers of AR knock out mice (ARKO) showed markedly reduced inflammation. While microglial activation remained the same, a significant reduction in macrophage-related cytokines was observed. Macrophages treated with Epalrastat, an AR inhibitor, showed reduced CNS inflammatory gene expression in response to LPS, indicating the role of AR in LPS-induced inflammation.

These studies implicate the GM in the CNS pathology of neurological-related diseases. The loss of butyrate has a critical role in the health of neurons and targeting this loss reduced or prevented neuropathology. Finally, we identified AR as a critical mediator of LPS-induced inflammation indicating that Epalrastat may aid in the treatment of CNS disease driven by endotoxemia.
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CHAPTER 1:

INTRODUCTION

The pathogenesis of neurological diseases, especially chronic diseases, are multifaceted and elements such as genetics, environment, age, and lifestyle etc. all have integral roles in the development of Alzheimer’s disease (AD), Parkinson’s diseases, and dementias. As we attempt to discern the factors associated with the development of neurological diseases and dementia, the gut-microbiome (GM) has recently been demonstrated as a previously unidentified, but highly influential, factor affecting neurologic function and pathology. With advances in technology, the ability to analyze commensal flora for their composition and structure as well as their metabolites has demonstrated mechanisms through which the GM interacts with the host to modulate disease progression or health. Dysbiosis of the GM leads to neuropathology and targeting the functional loses in the GM will ameliorate the pathological changes in the brain. This body of work seeks to provide data supporting this hypothesis. The gut-brain axis is emerging as a critical component of the health of the nervous system, and studies herein help to elucidate the role of the GM in neuropathogenesis by using two different models of neuroinflammation and a commonly used mouse model of early AD.

The Gut Microbiome

One of the most active research fields in human health and disease is the study of the GM, which is a dynamic system of bacteria communities starting in the oral cavity and continuing throughout the gastrointestinal tract (Dieterich, Schink, & Zopf, 2018). The GM is a widely diverse environment and, comprises 90% of the microbial cells in the
human body (C. H. Kim, 2013). These diverse communities are constantly being modulated by the environment, the host, and microbiome interactions (Schmidt, Raes, & Bork, 2018). The major phyla in the GM consists of Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia, with Firmicutes and Bacteroidetes making up 90% of the gut microbiota (Rinninella et al., 2019). These phyla can be disrupted causing an imbalance in the microbiota homeostasis leading to pathogenic alterations in functional composition and metabolism, which is known as dysbiosis (Tamboli, Neut, Desreumaux, & Colombel, 2004). The ratio of Firmicutes to Bacteroidetes (F/B ratio) is a crude but commonly used marker of dysbiosis. Indeed, an increase in the F/B ratio is associated with obesity and a decrease is associated with inflammatory bowel disease (S. F. Clarke et al., 2012; Stojanov, Berlec, & Strukelj, 2020). Bacterial communities within the GM play a major role in the synergistic relations with the host by digesting carbohydrates, fats, and other nutrients. In the process of metabolism, bacteria will then provide the host with products such as short chain fatty acids (SCFA) (C. H. Kim, 2013). Formate, acetate, propionate, and butyrate are the major SCFA produced by the bacteria (Morrison & Preston, 2016). When dysbiosis has become associated with a disease, interventions such as probiotics have been employed. Lactobacillus rhamnosus GG, a member of the Firmicutes, has been used clinically with varying success as a probiotic with the aim to shift the F/B ratio back towards Firmicutes when the ratio has been altered in metabolic disorders (Y. Liu, K. Chen, et al., 2020). The functional changes in the GM can lead to altered bacterial products that, in turn, can have a major effect starting locally in the intestine and ultimately effecting the brain of the host (Quigley, 2017).
The Gut-Brain Axis: How the gut affects the immune system and brain

Overview. The GM and immune system are tightly interconnected, with the latter dependent on the former for proper maturation and function (Q. Zhao & Elson, 2018). The GM directs the development of both the systemic immunity and the local immune system in the CNS. The local immune cells of the CNS can be activated either directly by the GM’s products or through indirectly activating the systemic immune system. The maturation process directed by the GM provides bacterial products as antigens to the immune system in order for it to recognize and target foreign entities that infiltrate the body. The maturation process is notably diminished in germ-free (GF) mice, who do not have a GM, which then leads to an exaggerated immune response to bacterial products indicating that the immune system is not mature without preexposure to the GM (Cammarota et al., 2015). The over-exaggerated response extends to the local environment in the CNS. Another connection is through the hypothalamic-pituitary-adrenal axis (HPA). In fact, the GM has been linked to pathogenic changes in the HPA axis that result in major depression, bipolar disorder, and schizophrenia (Misiak et al., 2020). These disorders have been linked to microbial products such as lipopolysaccharide (LPS) (Arioz et al., 2019). These pathways of the gut-brain axis are critical in the pathogenesis of various diseases, and we are now in the initial stages of understanding how relevant the GM is to specific diseases and the host’s reaction to various inflammatory stimuli.

Gut-barrier dysfunction in association with dysbiosis. The GM is contained within the lumen of the gut by a lining of epithelial cells that express large amounts of tight junction (TJ) proteins such as claudins, ZO-1, and occludin (Cresci, Bush, & Nagy, 2014; S. Guo, Al-Sadi, Said, & Ma, 2013). In conditions where the barrier has been compromised, LPS and other bacterial products seep into the peripheral circulatory system. Dysbiosis, a
shift of GM’s bacterial species composition, can be pathogenic leading to gut barrier
dysfunction (Cresci et al., 2014). Within the lumen of the intestines, LPS is at its highest
concentration in the body, but in the event of barrier dysfunction serum levels can
dramatically increase (S. Guo et al., 2013). After a pathogenic dysbiotic shift, bacterial
products such as SCFA can be dramatically altered (Cresci et al., 2014). A particular
SCFA of interest is butyrate or butyric acid. This 4-carbon fatty acid has a critical role in
maintaining the gut barrier (Peng, Li, Green, Holzman, & Lin, 2009). In studies done on
human intestinal cells, butyrate was able to facilitate TJ functionality by activating AMP-
activated protein kinase, providing evidence that butyrate is important in gut barrier
development and maintenance (Peng et al., 2009). A pathogenic dysbiotic state can
lead to the impairment of the gut barrier, which allows pathogen-associated-molecular-
patterns (PAMPs) and LPS to enter the circulation. Both PAMPs and LPS can act upon
toll-like receptors (TLR) to induce immune cell activation, pro-inflammatory cytokine
production and systemic inflammation (Tang, Kang, Coyne, Zeh, & Lotze, 2012). A more
porous barrier cannot protect the host from the proinflammatory bacterial products, can
induce constant low-grade inflammation, and can lead to systemic inflammatory state
changes which will then influence the state of the CNS.

Gut barrier protection by the resident immune cells. Resident macrophages prevent the
GM from escaping the barrier in the gut, preventing translocation of bacteria and their
by-products into the circulatory system (Medina-Contreras et al., 2011). These
macrophages are rich with CX3CR1 (fractalkine receptor) (Medina-Contreras et al.,
2011). CX3CL1 is produced by the epithelium to recruit and then promote dendrite
formation on native macrophages in the gut-associated lymphoid tissue (GALT) to
sample the GM (K. W. Kim et al., 2011). These CX3CR1 mature macrophages then
patrol the lamina propria for any foreign infiltration through the gut barrier (Bain &
The T cells within the lamina propria are rich in a population of γδ-TCR (T-cell receptor) T cells which produce large amounts of IL-17A to contain the GM. However, when the CX₃CR1 gene in macrophages is knocked out, the IL-17A response and bacterial translocation are exaggerated, providing the evidence for the importance CX₃CR1 in maintaining normal barrier function (Medina-Contreras et al., 2011). In diseases that disrupt gut barrier function, intestinal macrophages migrate to the site and are activated to produce cytokines such as, IL-12, IL-23, IL-1β, IL-6, and TNF-α, as well as the DCs that produce IL-6 and IL-23 (Cammarota et al., 2015). These cytokines then induce T cell differentiation into proinflammatory subsets of Th1 and Th17 (T helper), which produce IFN-ϒ and IL-17, respectively. The proinflammatory cytokines produced by the macrophages can then act on DCs, which then can migrate into the local lymph nodes to present antigens to activate T cells (Cammarota et al., 2015). The gut-mediated immune changes can cause a proinflammatory state in the intestine leading to systemic elevation of proinflammatory cytokines. The macrophages and T cells in the GALT play a critical role in maintaining gut barrier homeostasis and have a critical function in protecting the host.

**Microbiota-immune axis.** To determine the direct effects of the GM on the immune system, researchers initially utilized GF animals (Round & Mazmanian, 2009). Although the studies yielded striking results, subsequent and more thorough investigations indicated that the immature immune system and skewed immune responses in these animals were poor models for most experiments. However, results from these experiments still help define the impact of the GM on the immune system. In mice that are missing a GM, the adaptive immune system becomes dysregulated and stays immature, with a major disruption in the regulatory T cell population (Tregs) (Ostman, Rask, Wold, Hultkrantz, & Telemo, 2006). Tregs are important in maintaining immune
tolerance to autoantigens and innocuous exogenous antigens (Ostman et al., 2006).

With a noted deficiency in Tregs, the adaptive immune system reacts in an excessive manner. Not only are the Tregs under-expressed in the GF animals, but the functionality of those Tregs is dampened (Ostman et al., 2006). While Tregs that have been isolated from GF mice showed suppressed proliferation and function, their function was not completely lost (Ostman et al., 2006). GF mice also displayed a high frequency of naïve T cells as compared to their conventional counterparts and the T and B cells did not react normally to inflammatory stimuli, as shown by an exaggerated cytokine response (Ostman et al., 2006). The over reactive and skewed adaptive immune response is a confounding variable in any study performed in GF animals regardless of whether the GM is reconstituted in these animals.

**Indirect education of the T cells.** The GM produces metabolites that are absorbed by the host and educate the immune system. Retinoic acid (RA) is produced by bacteria that absorb retinol (C. H. Kim, 2013). While high levels are toxic, RA is an important regulator of T cell differentiation and function, inducing Treg polarization by acting through RA receptors which are expressed on naïve T cells (Kang, Lim, Andrisani, Broxmeyer, & Kim, 2007). RA also can act on dendritic cells (DCs) to induce Arg1, which further promotes the Treg phenotype in T cells (C. H. Kim, 2013). Through the release of RA into lumen of the gut, the GM is indirectly affecting the T cell population of the host system without directly presenting antigens.

**Direct modulation of the adaptive immune system.** The GM modulation of the adaptive system begins in the GALT, where antigen-presenting DCs sample the bacterial population residing within the lumen (Farache et al., 2013). DCs are part of the link between the innate and adaptive immunity and their main function is to present antigens to T cells. CD103-expressing DC, the resident DC in the intestine, patrol theintestinal
epithelium (Farache et al., 2013). Once drawn to an inflamed part of the epithelium by chemokines expressed after TLR signaling, DCs stretch pseudopodia through the gut barrier to acquire luminal samples of the GM (Cammarota et al., 2015; Farache et al., 2013). Chemokine (C-C motif) ligand 20 (CCL20), also known as macrophage inflammatory protein (MIP)-3α, is secreted by inflamed epithelial cells and has been putatively implicated in the chemoattraction of DCs to the barrier by acting on the CCL20 receptor Chemokine receptor 6 (CCR6) (Farache et al., 2013). Once DCs have sampled the GM, they in turn, produce interleukin 10 (IL-10), which prevents Th1 and Th17 differentiation and macrophage activation (Cammarota et al., 2015). DCs also present GM antigens to naïve B-cells to educate them to bind to these foreign pathogens for removal. Through these methods the GM is directly educating the immune system.

Taken together the GM influences how and to what degree the immune system will react to pathogens. The immune system’s reaction and cell polarization will have a significant impact on the CNS through cytokines, chemokines, and the activation state of immune cells that infiltrate the CNS. The beneficial and necessary responses of the immune system can become pathogenic and then lead to inflammation in the CNS and can disrupt the normal function of the brain (Fung, 2020). Therefore, a careful study of the gut’s effect on the immune system in diseases that cause dysbiosis is critical for understanding the pathogenesis of these conditions.

**GM induction of CNS Inflammation.** Within the CNS, astrocytes, and microglia play an important role in providing nutrients for neurons as well as participating in synaptic function, synaptic pruning and removing debris. However, as antigen presenting cells, astrocytes and microglia are integral in the immune response within the CNS. These functionalities may become maladaptive when prolonged activation occurs. GM products, particularly LPS, activate both astrocytes and microglia (Norden, Trojanowski,
Villanueva, Navarro, & Godbout, 2016). Due to its large size, it would be difficult for LPS to cross the blood brain barrier (BBB) without a shuttle. Through radioactive iodine labeling, it has been determined that only 0.025% of the intravenously administered LPS entered the brain parenchyma, and 75% of the BBB interactions with LPS were reversible allowing the LPS to be released from the BBB (Banks & Robinson, 2010). However, the mice used in the study were young at 8-12 weeks of age. Human MRI studies have found that aging increased the leakiness of the microvessels in the hippocampal region, which may provide an entry point in the CNS for LPS (Banks & Robinson, 2010). Regardless of whether LPS-driven activation within the CNS occurs directly or indirectly, microbial products and microRNAs will cause an inflammatory response by both peripheral and CNS immune cells (Fujihara et al., 2003; Nahid, Satoh, & Chan, 2011; Takeda & Akira, 2004). Systemic cytokines, such as TNF-α and IL-1β, can readily cross the BBB (Banks, 2005) at which point they can activate the resident cells in the CNS, specifically astrocytes and microglia.

Astrocyte involvement in CNS inflammation. In many neuropathological diseases, astrocytes, which are not immune cells, play an important role in the inflammation state of the CNS (Phatnani & Maniatis, 2015). Astrocytes, like macrophages, have polarization states that have been designated as A1 (proinflammatory) and A2 (anti-inflammatory). Stimulation with LPS+IFN-ϒ will induce an A1 state characterized by inducible nitric oxide synthase (iNOS) expression, a gene associated with the proinflammatory polarization of macrophages (Jang et al., 2013). By contrast, stimulation with IL-4 induces an A2 state distinguished by Arginase-1 (Arg-1) expression, a marker of anti-inflammatory states in macrophages (Jang et al., 2013). Astrocytes can be polarized towards A1 by the cytokines IL-1α and TNF-α along with C1q, all of which are released by activated microglia (Jang et al., 2013). Once polarized towards A1, co-culturing these
astrocytes with neurons caused 20% mortality in neurons, indicating they have a neurotoxic effect (Montagne et al., 2015). A1 astrocytes were worse at promoting new synapses and caused a decrease in the excitatory function of neurons when compared to A2 astrocytes (Jang et al., 2013). A1 polarization has now been shown to be significantly increased in post-mortem tissue from patients with AD, Huntington’s disease (HD), Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS), and Multiple Sclerosis (MS), stressing the importance of their role in the pathogenesis of neurological diseases (L. E. Clarke et al., 2018). In AD, 60% of the astrocytes in the prefrontal cortex, the brain area with the most neuronal damage, the expression of A1 phenotype is increased when compared to healthy controls (L. E. Clarke et al., 2018). A1 astrocytes are neurotoxic and may play a key role in the neurodegeneration seen in AD (Liddelow et al., 2017). To determine the time course of microglia and astrocyte activation, $Csf1r^{-/-}$ mice, which lack colony stimulating factor 1 receptors (a necessary receptor to support microglia) and conventional mice were treated with LPS to determine if microglia were activated astrocytes (Liddelow et al., 2017). The $Csf1r^{-/-}$ mice did not develop A1-polarized astrocytes providing evidence that microglia help activate astrocytes (Liddelow et al., 2017). Collectively, these results suggest that microglia activation in turn activates astrocytes towards an A1 polarization. While astrocytes are not considered immune cells, they can produce proinflammatory cytokines and play an important role in neuronal health. Therefore, astrocytes are critical in how the brain processes inflammation and their role in GM dysbiosis needs to be elucidated.

**Gut-driven microglia activation.** Microglia are the resident macrophages in the brain. They have a nominal phagocytic function, as well as a specialized function of interacting with neuronal synapses (Araque, Parpura, Sanzgiri, & Haydon, 1999). The GM has a strong effect on the morphology and maturation of microglia (Erny et al., 2015).
Structural morphology studies of microglia indicate that microglia from GF mice are less mature and do not space appropriately creating overcrowding (Erny et al., 2015). Immature microglia have a more ramified morphology, multiply faster and have exaggerated responses to LPS (Erny et al., 2015). To understand how the GM is acting on the microglia, specific pathogen-free mice were treated with broad-spectrum antibiotics for 4 weeks (Erny et al., 2015), and the microglia from these mice morphologically resembled microglia from GF mice, demonstrating that microglia revert to an immature phenotype with the repression of the GM (Erny et al., 2015).

Recolonization of GF mice with tri-Altered Schaedler Flora (ASF), a known flora with 3 bacteria strains, was unable to produce mature microglia, suggesting that a complex GM can properly mature the microglia (Erny et al., 2015). Since mice are coprophagous, co-housing ASF mice with conventional mice for 6-weeks restored a complex microbiota to the ASF mice and properly educated the microglia to morphologically resemble those of conventional mice (Erny et al., 2015). Mice deficient in the SCFA receptor, free fatty acid receptor 2 (FFAR2), also display an immature phenotype, indicating that SCFA help drive microglia maturation (Erny et al., 2015). To validate that role of SCFA, GF mice were treated with SCFA in drinking water, which led to a mature microglial phenotype (Erny et al., 2015). These findings provide strong evidence that SCFA are a link between the GM and CNS macrophages and this link requires SCFA likely produced by a complex microbiome (Erny et al., 2015). Interestingly, microglia do not express FFAR2, meaning SCFA must be acting in an indirect manner (Erny et al., 2015). These data indicate that the GM and its metabolites likely influence microglia function and dysbiosis could significantly affect the activation and maturity of microglia. These resident macrophages play a critical role in several diseases such as AD and PD, and GM regulation of microglia function will affect how they interact with disease-related proteins and could impact the severity of the diseases.
The GM drives changes in the hypothalamic-pituitary-adrenal axis. The GM can alternatively impact the HPA axis driven by a homeostasis imbalance. Physical, emotional, and immunological disturbances can trigger activation of the HPA and lead to a release of glucocorticoids (GCs). A dysbiotic GM can lead to increases of the pro-inflammatory cytokines, IL-6, TNFα, IL-1β, IL-17, IL-22 and IFNγ, which can be trafficked across the BBB (Banks, 2005; Schirmer et al., 2016). These cytokines can then drive an inflammatory response in the CNS which has been linked to depression (Harrison et al., 2009). In addition to cytokines, microbial products, activated immune cells, modulation of SCFA, and ileal corticosterone can all trigger the HPA axis activation (Misiak et al., 2020). Functionally, a prolonged or hyperactive HPA axis coupled with inflammation can also lead to depression (Stetler & Miller, 2011). Furthermore, fecal microbiota transplants (FMT) from depressed patients led to the development of depression-like symptoms in recipient mice (Evans et al., 2017). Interventions aimed at the alteration of the microbiome led to changes in brain-derived neurotropic factor (BDNF) expression in the CNS, in which increased levels of BDNF have been implicated in the reduction of depression (Bercik et al., 2011; Deltheil et al., 2008). Thus, the HPA axis is intimately linked to the state of the GM and should be considered when studying any disease that is associated with dysbiosis.

Proteostasis network and neuropathology

The proteostasis (cellular protein homeostasis) network (PN) accurately controls protein synthesis, folding, and degradation (Lottes & Cox, 2020) and is critical to normal functioning and health of a cell. An integral part of protein function is their appropriate folding conformation and the PN utilizes chaperon proteins to aid in the proper formation of proteins (Hartl, Bracher, & Hayer-Hartl, 2011). When misfolding or aging disrupts protein conformation, the ubiquitin proteasome system (UPS) and autophagy
mechanisms remove these cytotoxic proteins (Hartl et al., 2011). In addition to core PN mechanisms, the heat shock response (HSR), endoplasmic reticulum stress response (ERSR), integrated stress response (ISR) and the unfolded protein response (UPR) cooperatively attempt to restore protein homeostasis and, if restoration is impossible, initiate apoptosis (Costa-Mattioli & Walter, 2020).

**Heat shock response (HSR).** In response to increased temperatures, oxidative stress, and heavy metals, the HSR increases chaperon proteins to restore proteostasis (Morimoto, 1993). The upregulation of heat shock protein is mediated by the transcription factor heat shock factor 1 (HSF1) which is held inactive by chaperon proteins which release HSF1 in response to denatured proteins (Morley & Morimoto, 2004). HSF1 then translocates into the nucleus and trimerizes and binds to the promoter region of heat shock proteins to initiate their expression (Akerfelt, Trouillet, Mezger, & Sistonen, 2007) in attempts to restore protein homeostasis.

**Endoplasmic reticulum stress response (ERSR), unfolded protein response (UPR) and integrated stress response (ISR).** The ERSR, UPR, and ISR comprise overlapping signal modules which initiate both similar and distinct signaling cascades. The endoplasmic reticulum (ER) plays an important role in the PN as it is important for protein synthesis, folding and maturation. Aging or perturbations from disease can result in increased misfolding proteins (Kurtishi, Rosen, Patil, Alves, & Moller, 2019; W. Li et al., 2020), which initiate the ERSR to inhibit protein translation, increase folding capacity, and degradation of misfolded proteins (Ohri, Hetman, & Whittemore, 2013). Functionally, the ERSR comprises three signaling pathways: (1) protein kinase RNA-like ER kinase (PERK), (2) activating transcription factor (ATF6) and (3) inositol-requiring protein-1α (IRE1) pathways. The pro-homeostatic UPR is considered to encompass just ATF6 and IRE1 signaling. Functionally these 3 ERSR pathway proteins are inhibited by
constitutively bound glucose-regulated protein 78 (GRP78) which dislocates from each in response to the accumulation of misfolded proteins (M. Wang, Wey, Zhang, Ye, & Lee, 2009). Unbound ATF6 is translocated to the Golgi body where it is cleaved by site 1 (S1P) and site 2 (S2P) proteases to short form AFT6 (Ye et al., 2000). Once cleaved ATF6 functions as a transcription factor and promotes the expression of chaperon proteins, the pro-apoptotic transcription factor C/EBP homologous protein (CHOP) and X-box binding protein 1 (XBP1) (Yoshida et al., 2000). The activation of IRE1 leads to endoribonuclease activity and splicing of XBP1, which becomes a transcription factor upregulating chaperon proteins and genes related to protein degradation (Adams, Kopp, Larburu, Nowak, & Ali, 2019). Activated PERK signals through phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α) (Y. C. Wang et al., 2020), which inactivates global protein translation as specifically increases ERSR protein translation (Wek, Jiang, & Anthony, 2006). The ISR includes PERK, as well as general control nonderepressible 2, Protein kinase RNA-activated, and Heme-regulated eIF2α kinases, that all canonically signal through eIF2α. The interplay between these responses ultimately determines the restoration of homeostasis or apoptosis of the cell (Zeng, Li, Zhang, & Jiang, 2019). If unsuccessful, a sustained ERSR will hit a critical concentration of CHOP leading to apoptosis (Szegedzi, Logue, Gorman, & Samali, 2006).

Neuropathology and the ISR/ERSR/UPR. Many neurodegenerative diseases have been associated with the misfolding and aggregation of proteins. Alzheimer’s disease (Aβ/P-tau) (AD), Parkinson’s disease (α-synuclein), Huntington’s disease (Huntingtin), and amyotrophic lateral sclerosis (ubiquitinated proteins) are classified in part by the proteins that aggregate (Barker, Fujimaki, Rogers, & Rubinsztein, 2020; Ghemrawi & Khair, 2020; Ross & Poirier, 2004). The accumulation of these protein aggregates enhances degeneration of neurons resulting in cognitive decline. The ability to restore protein
homeostasis to neurons is diminished and eventually they undergo apoptosis (Scheper & Hoozemans, 2015). The robustness of the evidence strongly connects the ERSR with neurodegenerations and implicates it as therapeutic target.

**Ethanol as a pathogenic driver of dysbiosis and inflammation**

**Ethanol driven dysbiosis.** Ethanol consumption is a powerful modulator of the GM and the environment of the intestines. Indeed, individuals who consume ethanol for a prolonged period experience extreme dysbiosis (Singhal et al., 2021) characterized by a loss of butyrate-producing capacity which is critical to the intestinal integrity and the GM (Singhal et al., 2021), ethanol not only modulates the GM composition, but also affects the host’s gut barrier. Both ethanol and its metabolite acetaldehyde disrupt TJ proteins in the gut epithelium (Elamin et al., 2012). Butyrate builds up the mucosal layer, increases expression of TJ proteins and has anti-inflammatory effects, indicating the importance of butyrate to maintaining the health of the gut barrier (Baxter et al., 2019; Inan et al., 2000; Yan & Ajuwon, 2017). In recent work, it was shown that chronic ethanol feeding led to a reduction in microbial diversity (Singhal et al., 2021). Ethanol feeding in a mouse model caused a substantial decrease in the largest butyrate-producing family, *Lachnospiraceae*, with 36 distinct butyrate-producing genera reduced (Singhal et al., 2021). Ethanol-mediated dysbiosis in these mice was characterized not only by a loss of butyrate producers but also a significant increase in both frequency and number in the phylum Proteobacteria (Singhal et al., 2021). This phylum consists of gram-negative bacterial species which are a primary source of intestinal LPS and systemic endotoxemia (Bull-Otterson et al., 2013). Collectively, these data show that changes to intestinal integrity and pathogenic dysbiosis mediated by ethanol consumption drive inflammation in the intestine.
Gut-Liver-Brain axis. Much of the circulating blood from the intestine is transported through the hepatoporal vein, making the liver the initial site for clearing gut microbial toxins from the blood. Dysbiotic microbiome-driven inflammation has been linked to the development of liver damage (Donde et al., 2020). Increased gut permeability leads to endotoxemia, a highly inflammatory state which may lead to sepsis (Bull-Otterson et al., 2013; Rao, Seth, & Sheth, 2004). This hepatic inflammation is characterized by Kupffer cell (liver resident macrophages) activation, neutrophil infiltration, and increased pro-inflammatory cytokine expression (Eguchi, McCuskey, & McCuskey, 1991; Ramaiah & Jaeschke, 2007). Prolonged neutrophil infiltration, immune cell activation and inflammation lead to hepatocyte death and progression of liver disease (Brenner, Galluzzi, Kepp, & Kroemer, 2013). These highly inflammatory events will contribute to systemic inflammation that will impact brain. Indeed, hepatic encephalopathy (HE) is a disease in which liver damage causes patients to develop neurological symptoms from an inability to dispose of GM toxins (Rocco et al., 2021). Thus, the liver is a potential mediator in the gut-brain axis.

CNS effects of ethanol consumption. Both clinical and preclinical data show that consumption of ethanol leads to neuroinflammation and neurodegeneration (Crews et al., 2004; Topiwala et al., 2017). In preclinical models, gut and liver inflammation lead to neuroinflammation (Crews et al., 2004; Szabo & Lippai, 2014). One of the defining pathologies of ethanol consumption models is microglial activation shown by increased ionized calcium-binding adapter molecule 1 (IBA-1) concentrations, which are similar to that seen in post-mortem human samples with alcohol use disorder (AUD) (J. He & Crews, 2008; Lowe et al., 2020). Microglial activation provides a critical function of protecting the host from pathogens, but with prolonged or overactivation, it can become maladaptive (Santiago, Bernardino, Agudo-Barriuso, & Goncalves, 2017). Ethanol has a
direct effect on the activation state of microglia and acts in part through TLR4 (Fernandez-Lizarbe, Pascual, & Guerri, 2009). Once activated, microglia can become chronically activated and remain so for years following a single insult (Langston et al., 1999). Prolonged activation can result in damage to dopaminergic neurons and may contribute to neurodegenerative diseases (Gao et al., 2002). Indeed, in comparison to the liver, the brain seems to have a limited capacity to mount an anti-inflammatory and immunosuppressive IL-10 response to attenuate the activation state of microglia (Qin et al., 2008). Taken together, ethanol plays a role in the inflammation state of the microglia through direct and indirect pathways and when paired with dysbiosis may further exacerbate CNS neurodegeneration.

Alzheimer’s Disease: a disease of the gut microbiome?

AD is one of the most common forms of dementia and is generally characterized by memory loss and a disruption of cognitive abilities. Amyloid-β (Aβ) plaques and neurofibrillary tangles abnormally build up in the brains of these patients causing neuronal damage (Vogt et al., 2017). Clinically, AD patients experience dysbiosis characterized by a decrease in richness and diversity of bacteria in the GM (Vogt et al., 2017). Specifically, there is a decrease in the phylum Firmicutes, which is similarly decreased in type 2 diabetes and obesity (Vogt et al., 2017). In particular, the specific Firmicutes families that were found to be decreased in AD patients were Ruminococcaceae, Turicibacteraceae, Peptostreptococcaceae, Clostridiaceae, and Mogibacteriaceae, and the genera SMB53 (family Clostridiaceae), Dialister, Clostridium, Turicibacter, and cc115 (family Erysipelotrichaceae) (Vogt et al., 2017). The AD patients also have an increase in the phylum Bacteroides, as the families Bacteroidaceae and Rikenellaceae were more abundant in AD participants, resulting in an increase in the F/B ratio (Vogt et al., 2017). Bacteroides include an abundant number of gram-negative
bacteria, which release pro-inflammatory LPS (Vogt et al., 2017). Of particular importance, the dysbiosis associated with the AD patients described above correlated with the cerebral spinal fluid (CSF) levels of AD-associated markers such as phosphorylated tau (P-tau) and amyloid-β peptides (Vogt et al., 2017). Corroborating work done in *Drosophila* indicates that gut dysbiosis can advance the progression of AD by increasing neuroinflammation and monocyte recruitment to the brain (Wu, Cao, Chang, & Juang, 2017). As previously stated, a pathogenic dysbiotic state can also induce gut barrier dysfunction and systemic endotoxemia. Interestingly, when administered peripherally, LPS can cause neuroinflammation and when administered chronically can result in accumulation of Aβ proteins in the hippocampus (Wu et al., 2017). The evidence is mounting that the inflammatory response to the pathogenic state of the GM is a leading factor in the progression of AD and must be considered when determining therapeutic interventions of patients with dementia.

**Glia cell activation and immune cell infiltration in AD.** In AD, the microglia shift towards a proinflammatory M1 phenotype due to an increase in Aβ plaque buildup in both patients and mouse models (Varnum & Ikezu, 2012). M1 microglia release proinflammatory cytokines and induce A1 polarization in astrocytes, both of which, as detailed above, can become neurotoxic. One of the defining features of AD inflammation is Aβ oligomers (ABO) causing an inflammatory response (Balducci et al., 2017). Injecting ABO into the brains of mice yielded microglia and astrocyte activation peaking at 8 hours, and, as a result, IL-1β was mainly expressed in astrocytes and not microglia, indicating the IL-1β expressing astrocytes may play a more important role in the pathogenesis of AD (Balducci et al., 2017). Protein measurements from the brains of the AD model where mice were treated with ABO showed an increase in additional proinflammatory cytokines and chemokines IL-6, CCL5, TNF-α, MIP-1α, and monocyte chemoattractant protein
Importantly, inflammation driven by ABO acts through TLR4, providing strong evidence that Aβ acts as a highly inflammatory TLR ligand (Balducci et al., 2017). Degenerating neurons further contribute to inflammation through release of High mobility group box 1 (HMGB1) (Fujita et al., 2016). This danger associated molecular pattern (DAMP), can bind to amyloid-β monomers enhancing oligomerization and can enhance TLR4 activation by binding to itself or with increased Aβ oligomers (Fujita et al., 2016). Collectively, these data point to AD being a chronic neuroinflammatory state.

**Adaptive immune system in AD.** The inflammation includes infiltration from the adaptive immune system (Laurent et al., 2017). In the THY-Tau22 AD model, transgenic mice that overexpress human four-repeating tau demonstrated an increase in T cell infiltration in the hippocampus (Laurent et al., 2017). Specifically, cytotoxic CD8 T cell infiltration and inflammation could be reversed with functional grade anti-CD3 treatment, providing evidence that T cells play an important role in AD neuroinflammation (Laurent et al., 2017). Thus, both infiltrating immune cells and the resident glial cell activation seem to play a significant role in AD.

**Rodent models of AD and dysbiosis.** The most widely used models for studying the development and progression of AD are the 5xFADs and 3xTgs. The 5xFAD model employs 5 human genetic polymorphisms to induce AD-like pathology quickly. By 6 months, the 5xFAD mice have developed large and dense amyloid-β plaques in the hippocampus and exhibit cognitive decline. However, these mice do not develop tau pathology. As expected, amyloid-β plaques drive inflammation which, in turn, activates both microglia and astrocytes (Mirzaei et al., 2016). More interestingly, 5xFAD mice have been shown to develop dysbiosis (Lee, Lee, Kim, & Kim, 2019). Indeed, when the 5xFAD mouse’s microbiome is transplanted into non-transgenic mice, microglia
activation and pro-inflammatory cytokine expression were observed and the recipient mice developed cognitive decline (N. Kim et al., 2021). Further evidence on the importance of the GM, comes from another study that indicated that a targeted probiotic treatment reducing the AD-like pathology in 5xFAD mice (Guilherme, Nguyen, Reinhardt, & Endres, 2021; Lee et al., 2019). These recent studies suggest the AD-like pathology in these mice is linked to the microbiome.

3xTg mice have three genetic modifications: an insertion of APP (Swedish mutation), a microtubule associated protein tau (MAPT) prone to phosphorylation, and a loss of function of presenilin 1 which causes incomplete digestion of amyloid-β. This model more fully mimics the development of AD because the mice develop neurofibrillary tangles. They begin showing signs of cognitive decline around 4 months of age, amyloid-β pathology at 6 months, and tau pathology at 12 months. Much like 5xFAD mice, 3xTg mice display CNS neuroinflammation characterized by microglial activation (Chen et al., 2014). However, these mice do not present with the AD-like astrocyte activation and their activation is believed to be due to aging (Bronzuoli et al., 2019). The most important difference between the 5xFAD and 3xTg models is the development of tau pathology. As part of the 3 transgenes, the MAPT gene makes the expressed tau susceptible to hyperphosphorylation. Microglia activation associated with amyloid-β plaques have been linked to the propagation of P-tau, through phagocytizing neurons with P-tau and the release of vesicles containing prion-like P-tau (Clayton et al., 2021). Following a similar trend with the age-related development of AD-like symptoms is the onset of GM dysbiosis (Bello-Medina et al., 2021). In fact, longitudinal studies indicate that the dysbiosis is characterized by a loss of diversity (Bello-Medina et al., 2021). Treatment of 3xTg mice includes a probiotic diet, lysates of Bifidobacterium and Lactobacillus acidophilus, vitamins A and D, and w3 fatty acid from cod-liver oil, as well
as vitamins B1, B3, B6, B9, and B12. This diet partially ameliorates cognitive decline in these mice (Tan et al., 2022). Thus, in both 3xTg and 5xFAD mice, changes in the GM play a major role in the observed neuropathology.

**Summary of experimental directions**

Hence, to determine the role of GM in the pathogenesis of both ethanol-induced neuropathology and AD, several strategies will be employed. To determine the direct role of the microbiome in ethanol-related neuropathology, a novel FMT model in conventional mice was employed crucially without the use of antibiotics. Specifically, GM from patients with alcohol associated hepatitis patients was characterized by 16S rRNA gene sequencing and whole genome shotgun (WGS) metagenomic sequencing. The well characterized microbiome was then transplanted into C57/BL6J mice whose GM was purged and a targeted therapeutic approach was utilized to restore lost metabolites and facilitate functional recovery.

3xTg mice were used to determine if or how dysbiosis plays a role in the temporal sequelae of AD pathogenesis. Once again, 16S rRNA gene sequencing and WGS metagenomic sequencing characterized the microbiome. Then, any functional loss in the microbiome was targeted serving the dual purpose of providing further evidence of the role of the GM as well as a being a potential therapeutic strategy in the treatment of AD.

Finally, determine a possible gene through which GM products drive systemic inflammation and lead to neuronal inflammation have been identified, and administration LPS injection intraperitoneally which will drive TLR4 activation, a known mechanism leading to CNS inflammation in a variety of situations including dysbiosis-driven and AD
pathology-driven neuroinflammation and assess the effects on the CNS with our gene of interest.
CHAPTER 2:
ETHANOL-INDUCED DYSBIOSIS IS CRITICAL IN THE PATHOGENESIS OF RELATED NEUROPATHOLOGY

Introduction

The mammalian intestine harbors the gut microbiome (GM), a vibrant microbial community that permanently resides in a symbiotic relationship with the entire organism and is the major producer of pathogen-associated molecular pattern molecules (PAMPs) which act as inflammatory stimuli to the immune system (Mogensen, 2009). The intestines have mechanisms that control the release of PAMPs and other inflammatory agents into the systemic circulation. For example, lipopolysaccharide (LPS/endotoxin, a bacterial wall inflammatory molecule) concentrations in the blood of normal rats and healthy individuals are barely detectable because of sustained barrier impermeability (Fukui, Brauner, Bode, & Bode, 1991; Mathurin et al., 2000; Parlesak, Schafer, Schutz, Bode, & Bode, 2000).

The GM fermentation of insoluble fibers provides the primary source of short-chain fatty acids (SCFA), the most prominent of which are acetate, butyrate, and propionate. Butyrate has anti-inflammatory properties, helps to maintain mucus in the intestines, acts as a histone deacetylase (HDAC) inhibitor as well as increases epithelial barrier proteins (H. Liu et al., 2018). The GM forces maturation and dictates the status of the immune system, particularly regarding central nervous system (CNS) microglia, whose maturity and activation status is directly related to the GM and butyrate production (Erny et al., 2015). There exists a synergistic relation between the GM and CNS inflammation which is related to butyrate concentrations, and various CNS
pathogenic states are correlated with disrupted butyrate synthesis (Morrison & Preston, 2016). Importantly, dietary butyrate supplementation can attenuate dysbiosis of the microbiome which leads to chronic neuroinflammation (Jackson et al., 2016; Matt et al., 2018).

Microbiome dysbiosis has been linked to several CNS diseases, including Alzheimer’s disease (AD), Parkinson’s disease (PD), chronic metabolic disorders and depression (Rogers et al., 2016; Sampson et al., 2016; Wu et al., 2017). Ethanol induces pathogenic alterations in the GM that can cause gut barrier dysfunction which increases gut permeability and translocation of whole bacteria or bacterial products leading to endotoxemia (Bull-Otteron et al., 2013; Rao et al., 2004). Ethanol-mediated dysbiosis in mice is characterized by significant increases in the phylum Proteobacteria. This phylum consists of gram-negative bacterial species that are a primary source of intestinal endotoxins and systemic endotoxemia (Bull-Otteron et al., 2013). Similarly, ethanol also compromises tight junctions of the BBB leading to increased CNS permeability (A. K. Singh, Jiang, Gupta, & Benlhabib, 2007). While the evidence for the gut-brain axis is well established (Carabotti, Scirocco, Maselli, & Severi, 2015), the causal role of an ethanol-modified GM on neuroinflammation and neurodegeneration has yet to be determined.

The pathological effects of ethanol-related neuroinflammation and neurodegeneration have been documented in the prefrontal cortex, hippocampus, and cerebellum of patients with alcohol use disorders (AUD) (Agartz, Momenan, Rawlings, Kerich, & Hommer, 1999; Nagel, Schweinsburg, Phan, & Tapert, 2005; Wilkinson & Carlen, 1980; Wilson, Bair, Thomas, & Iacono, 2017). Chronic alcohol abuse can result in patients with impaired or modified executive function and is considered to be more dangerous than cocaine abuse in that regard (Agartz et al., 1999). AUD patients can present with reduced prefrontal cortex gray matter as well as white matter (Gropper et al., 2016; Loheswaran et al., 2017; X. Yang et al., 2016). These pathologies are
associated with chronic, as opposed to binge, drinking (Waszkiewicz et al., 2018). Post-mortem analysis of prefrontal cortex from AUD patients has shown decreased cell numbers (Abernathy, Chandler, & Woodward, 2010), indicating that cortical volume loss is due to loss of cells. The damage done to the prefrontal cortex can lead to addictive-like behavior and is a critical component of CNS pathology and drug-seeking behaviors (Jentsch & Taylor, 1999; Kalivas, 2009).

There are reports of a decreased volume in the hippocampus in both adolescents and adults with AUD (Agartz et al., 1999; Nagel et al., 2005; Wilson et al., 2017). These findings indicate learning and memory impairments in subjects with AUD who present with hippocampal volume reduction. Indeed, reduction in hippocampal volume correlates with a reduction in anterograde memory and may play an important role in the development of dementia (Wilkinson & Carlen, 1980). The decrease in volume of the hippocampus is proportional to the decrease in volume of the rest of the brain, memory and learning impairments are present in these patients (Agartz et al., 1999).

Ethanol disruption and damage to the CNS particularly affect neurons in the hippocampus and cerebellum. In the cerebellum, Purkinje cells (PC) function by regulating and coordinating movements and they also aid in cognitive processing and sensory discrimination. Damage to the PCs leads to ataxia as well as deficient motor learning and impaired behavioral control (Hoxha, Balbo, Miniaci, & Tempia, 2018). Chronic alcohol consumption can lead to a decrease in the number of PCs (Andersen, 2004; Baker, Harding, Halliday, Kril, & Harper, 1999). Furthermore, there is evidence that moderate chronic alcohol consumption leads to a loss of PCs and cerebellar atrophy (Karhunen, Erkinjuntti, & Laippala, 1994). Thus, hippocampal and cerebellar neurons are particularly sensitive to ethanol toxicity. The negative neuronal effects are not well documented in the murine models of ethanol consumption because the relevant models do not recapitulate the human like pathology.
The goal of these studies was to determine whether alcohol-induced injury to the gut-brain axis leads to CNS inflammation and neuronal damage in a short-term, chronic alcohol feeding mouse model when mice are transplanted with an alcohol-driven dysbiotic GM from human subjects with severe alcoholic hepatitis. A causal role of an ethanol-experienced human GM in the development of neuroinflammation is shown and we describe a mechanism for degeneration of both granule and pyramidal neurons of the hippocampus and PCs in the cerebellum.

**Methods**

**Animal model:**

11-week-old male C57BL/6 were obtained from Jackson Laboratory (Bar Harbor, ME) (n=9-15) with a total of 3 studies. All mice were housed in a pathogen-free, temperature-controlled animal facility with 12-hour light /12-hour dark cycles. All experiments were carried out according to the criteria outlined in the Guide for Care and Use of Laboratory Animals and with approval of the University of Louisville Institutional Animal Care and Use (IACUC) and Institutional Biosafety (IBC) Committees.

**Fecal microbiome transplantation:**

To assess the role of an ethanol-experienced microbiome on the development of neuroinflammation and neurodegeneration, we developed a model of FMT that ingrafts the microbiome of a patient with AUD into mice. All mice were fasted overnight, before treatment with polyethylene glycol (PEG) 4000. The PEG 4000 was dissolved in water to a concentration of 425 g/L and administered by gavage - four times with 20 minutes rest between each treatment. Four hours after the final PEG 4000 treatment, FMT samples (1 g/20 mL) composed of the patients who had the top 3 MELD scores, in brain heart infusion media (37 g/L) were administered to mice by oral gavage. Mice received FMT 3
times in the first week and 2 times a week in the subsequent weeks until the termination of the experiment.

**Chronic ethanol consumption model:**

Mice were acclimatized to the Liber DeCarli diet with no ethanol for 5-7 days. In the following week, mice were separated into alcohol-fed (AF) and pair-fed (PF) groups. Pair feeding was iso-calorically controlled by matching the amount consumed of ethanol in the AF group the previous day using maltose dextrin which when metabolized has the same caloric content as ethanol. The AF groups were slowly ramped from 1% to 5% (v/v) ethanol over the course of a week followed by two consecutive weeks of 5% ethanol.

**Supplementation of butyrate**

Oral supplementation of tributyrin, a butyrate pro-drug, was gavaged into the mice 5 days a week at dose of 5 mg/kg

See Figure 1 for a graphical representation of chapter 2 experimental design

**Analysis of Butyrate by Gas Chromatography–Mass Spectrometry**

Fecal/cecal samples were derivatized with pentafluorobenzyl bromide and extracted with hexane. The hexane extract was analyzed by gas chromatography–mass spectrometry using an Agilent DB-225 J&W GC column (Agilent Technologies Inc, Santa Clara, CA) with a temperature gradient from 50°C to 220°C at 30°C/min, at a flow rate of 1.5 mL/min with helium as a carrier gas. Detection was by electron ionization and selective ion monitoring of m/z at 256 for sample and m/z at 257 for 13C-labeled butyrate standard. Using the peak area for corresponding labeled and unlabeled derivatives, a standard curve was generated by plotting 12C/13C ratios against the concentration of solutions. The slope and intercept were used to determine the concentration of butyrate in the samples.
Immunohistochemistry:

Mice were transcardially perfused with 20 ml PBS, followed by 15 ml of 4% formaldehyde (PFA) in PBS, pH 7.4. Brains were dissected and additionally fixed for an hour at 4°C in 4% PFA. Following fixation, brains were transferred to 30% sucrose solution and stored for 3 days at 4°C. Following sucrose dehydration, half of the brain was used for paraffin embedding and the other was cryopreserved. The tissue was embedded in freezing media (Triangle Biomedical Sciences, Durham, NC) sectioned coronally at 30 μm on a cryostat, then mounted on microscope slides (Fisher Scientific, Pittsburgh, PA) and stored at −80°C. Slides were warmed at 37°C for 20 minutes and tissue freezing media removed. For paraffin, after embedding tissue was sectioned 10 μm thick. After sectioning and mounting to slides, the slides were incubated at 37°C for 48 hours. For paraffin embedded tissue, paraffin removal, hydration, and antigen retrieval, slides were immersed in trilogy (Millipore Sigma, St. Louis, MO) and incubated in a pressure cooker at high pressure for 12 minutes. Both paraffin and cryo-embedded tissues were blocked in TBS + 0.1% Triton X-100, 0.5% BSA, and 10% normal donkey serum for 1 hour at room temperature and then incubated overnight at 4°C with primary antibodies in blocking buffer, followed by incubation in secondary antibodies at room temperature for 1 hour. The slides were imaged on a Keyence BZ-X810 (Keyence Corporation of America, Itasca, IL). Fluorescence intensities were measured with BZ-X810 analysis software (Keyence Corporation of America, Itasca, IL). Serial sections (n=3-5) of brain tissue were used to determine an average value for each animal. Values were only removed for damaged or folded tissues that prohibited accurate analysis. For each antibody, an isotype and no primary were used as controls. See data table 2 for antibody information.

Mononuclear cell isolation: Brains were harvested from mice transcardially perfused with 20 ml PBS, washed in 1 mL of PBS, minced in Liberase LT in HBSS with Ca2+ and
Mg2+ (Millipore Sigma), and incubated at 37°C for 30 minutes. Tissue was then forced through a 70 μm filter and washed with 3 mL PBS+10% FBS with DNase followed by 10 mL of PBS+10% FBS. Myelin was removed using 33% Percoll plus (Millipore Sigma) and washed with 10 mL of PBS+10% FBS.

**Flow Cytometry:**

Brain cell suspensions were incubated for 5-10 minutes with 1 μg/sample anti-CD16/32 mAb (clone 2.4G2, BioLegend, San Diego, CA) to block non-specific Ab binding to FcR (FcγR III/II), then stained with fluorochrome-labeled antibody panels. Cells were then washed in FACS staining buffer (SB: 1X PBS containing 2% FBS and 0.02% sodium azide) then 1X PBS and fixed in 2% methanol-free formaldehyde (Polysciences, Warrington, PA) for 2-24 hours before acquisition on a BD FACSCanto II flow cytometer, (BD Biosciences, CA). The following monoclonal antibodies were used: CD11b (Clone: M1/70), FITC, eBioscience; CD45 (30-F11), PerCP-Cyanine5.5, eBioscience; Ly-6G (1A8-Ly6g), PE, eBioscience; and Ly-6C (HK1.4), PE-Cyanine7, eBioscience). Panel-specific fluorescence minus one (FMO) controls on spleen cell populations were used to define negative events in gating strategies (Maecker & Trotter, 2006).

**Cytokine analysis:**

Brain tissues were homogenized in a buffer containing 20 mM Tris, 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, 1% Triton X-100, and a cocktail of protease inhibitors (Sigma) (Fox et al., 2005). The levels of various cytokines/chemokines were measured using MSD (Meso Scale Diagnostics, Rockville, MD) kits.

**Metagenomic Analysis**

Genomic bacterial DNA was extracted from fecal samples using MagAttract PowerSoil Kit (Qiagen). 16S rRNA gene sequencing methods were adapted from the NIH-Human Microbiome Project (Human Microbiome Project, 2012a, 2012b). The 16S rDNA V4 region is amplified by PCR using primers (GGACTACHVGGGTWTCTAAT and
GTGCCAGCMGCGCGGTAA) that contain adapters for MiSeq sequencing and dual-index barcodes so that the PCR products may be pooled and sequenced directly targeting minimum 10,000 reads per sample (Caporaso et al., 2012; Caporaso et al., 2011). Amplicon libraries were purified using SPRI beads on a Biomek liquid handler. DNA was quantitated by Quant-iT PicoGreen assay, normalized, pooled and followed by large-scale parallel sequencing on the Illumina MiSeq platform using the v2 SBS chemistry. An operational taxonomic units (OTUs) table was generated by taxonomic binning via the 1.91 version of Quantitative Insights into the Microbial Ecology (QIIME) (Caporaso et al., 2010) and its various components (Caporaso et al., 2010; DeSantis et al., 2006) using default settings unless otherwise noted. Closed reference OTU picking was performed using OTUs with 97% similarity to the clustered 13_8 release of the Greengenes reference database packaged with QIIME (McDonald et al., 2012). To maximize the sensitivity of detecting microbial taxa, the sequence read after quality filtering was inputed into the RDP Classifier (Q. Wang, Garrity, Tiedje, & Cole, 2007) to generate a table of taxonomic abundances in each sample. Default settings were used with the RDP Classifier except the confidence cutoff which was set at 0.5 and the format which was set as fixrank. Further QIIME 1.91 pipeline generated output for alpha- and beta-diversity and provided taxonomic summaries that were leveraged for all subsequent analyses.

**PICRUST analysis**

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013) was used for predictive analysis of the butyrate synthesis pathway. This computational approach uses 16S rRNA marker gene data in combination with a table of gene copy numbers of enzyme families present within each sequenced archaeal and bacterial taxonomic group in the IMG (Integrated Microbial Genomes) database. The output consists of the counts of coding genes from the Kyoto
Encyclopedia of Genes and Genomes (KEGG). PICRUSt was also used to generate output showing the contribution of each OTU to each KEGG gene. Default settings of PICRUSt were used except during the generation of OTU contributions to genes where only butyrate synthesis coding genes were retrieved.

Statistical Analysis

All statistical tests were performed using nonparametric tests: Mann U Whitney test, Kruskal Wallis test with corrections (Dunn’s multiple comparisons adjusted p-value or Bayesian q-value)(Carvajal-Rodriguez & de Una-Alvarez, 2011), and Spearman correlations.

Results

Demographic and clinical characteristics of the study population:

A pilot human study was conducted to evaluate differences in gut microbial composition from clinically diagnosed patients with alcoholic associated hepatitis (AH) (n=13) compared with age and gender matched healthy control (normal) participants (n=9). The demographics of healthy controls and AH study subjects are shown in Table 1. There were no significant differences seen in an age, sex, or racial distribution between the control and AH study populations. The patients with AH who enrolled in this study had a mean MELD (Model for end-stage liver disease) score of 22.08±6.12 and a mean MDF (Maddrey's discriminant function) score of 55.92±29.93.

Metagenomics analysis was performed on fecal samples obtained from patients with AH and control normal individuals.

A significant dysbiosis in patients with AH as compared to the normal subjects was demonstrated by a decrease in the alpha diversity measures such as the Shannon Index and Chao1 Index (p <0.0001) (Fig. 2 A,B). Notably, similar to our findings in mice exposed to chronic ethanol, patients with AH showed a marked decrease in the two
largest butyrate-producing families Lachnospiraceae and Ruminococcaceae (Fig. 2 C,D). Further analysis of the butyrate-producing families led to identification of 37 distinct butyrate-producing genera. Within the butyrate-producing genera, a significant loss of 23/37 genera was observed in Patients with AH as compared to normal subjects (Fig. 2,E). These results indicate that loss of butyrate-producing bacteria is a pathogenic feature of alcohol-induced microbial dysbiosis that occurs in alcoholic hepatitis patients and could potentially play a causal role in the development of alcoholic liver disease (ALD).

**Development of a mouse model of human fecal microbiota transplantation (FMT) into mice:**

To establish the causal role of gut bacteria dysbiosis into the development of ALD disease pathogenesis, we developed the innovative mouse model where the fecal microflora from Patients with AH was transplanted into the conventional mice. Fecal specimens from patients with AH (AH-FMT) with the characteristics described before, along with appropriate healthy normal control subjects (nFMT) were used to transplant fecal microbiome into conventional C57BL/6 mice. See figure 1 for a graphic representation for the experimental design.

**Fecal microbiota transplant from patients with AH into mice leads to a decrease in gut microbial diversity.**

The alpha diversity Shannon Index measuring species abundance and evenness was examined in the PF, PF+N-FMT, and PF+AH-FMT groups as well as AF, AF+N-FMT, and AF+AH-FMT groups. The data showed that in comparison to control PF and AF groups, there was a decrease in alpha diversity in both PF+AH-FMT and AF+AH-FMT (Fig. 3A). In addition, PF+AH-FMT and AF+AH-FMT groups also showed a significantly lower diversity as compared to PF+N-FMT and AF+N-FMT groups.
Interestingly, AF-N-FMT had a significantly higher diversity as compared to the AF group (Fig. 3A). These data affirm that FMT from Patients with AH and normal healthy controls not only altered the mice microbiome but also mirrored a similar decrease in diversity in AH-FMT groups and increase in diversity in nFMT groups as observed in the human population. Overall, the predictive alpha diversity measures indicate that FMT from Patients with AH leads to a decrease in microbial diversity.

Further, beta diversity was assessed to examine the dissimilarity of the microbial taxa between the treatment groups, using the phylogeny-based Un-weighted Unifrac distance metric PCA plots (Fig. 3B, C). The plot reveals that PF+AH-FMT and PF+N-FMT form a distinct cluster as compared to the PF group. Similarly, AF+AH-FMT and AF+N-FMT form a distinct cluster as compared to the AF group. These data indicate that FMT groups have altered microbiome composition as compared to control groups (Fig. 3B, C). The plot also reveals that between FMT groups, PF+AH-FMT and PF+N-FMT are two distinct clusters and AF+AH-FMT and AF+N-FMT are two distinct clusters, further suggesting compositional differences between N-FMT and AH-FMT. Therefore, fecal microbiota transplant from Patients with AH leads to distinctive changes in microbial composition.

**Fecal microbiota transplant from patients with AH is characterized by a decrease in butyrate-producing bacteria**

Our earlier findings in mice and the human patients with AH showed that alcohol use leads to a significant reduction in butyrate-producing communities (Fig. 2 E). Since the phylum *Firmicutes* harbors the majority of butyrate producers, we further extended taxonomic analysis specific to *Firmicutes* and its further classifications. Accordingly, our taxonomic study revealed that FMT from Patients with AH led to a significant drop in the families, *Lachnospiraceae* and *Ruminococcaceae* in the PF+AH-FMT and AF+AH-FMT
groups as compared to PF and AF groups respectively (Fig. 3 E-H). We also observed that FMT from normal healthy individuals led to significant increase in the families *Lachnospiraceae* and *Ruminococcaceae* families for both PF+nFMT and AF+nFMT groups as compared to PF+AH-FMT and AF+AH-FMT groups respectively (Fig. 3 A). This result corroborates our finding that alcohol use leads to significant loss of *Firmicutes*. Specific examples include the two largest butyrate-producing families, *Lachnospiraceae* and *Ruminococcaceae* (Fig. 2 C-E). These results indicate that microbial dysbiosis that includes a loss in butyrate-producing families seen in Patients with AH was established in the FMT model. Additionally, FMT from normal individuals not only prevented this loss but also led to increases in several butyrate-producing families.

Overall, these results not only validate our findings in mice and human subjects but also reveal that the loss of butyrate-producing communities plays a causal role in the development of alcoholic liver disease. Therefore, we conclude that reestablishing butyrate producers (as observed in nFMT groups) can mitigate the alcohol-induced microbial dysbiosis.

**Transplantation of AH patient microbiome drives systemic and CNS inflammation.**

In the purged conventional recipient mice, transplantation of fecal microbiome from AH-patients led to the induction of increased frequency of PMNs defined as CD45+/CD11bhi/Ly6G+ in both the spleen (from 1% to 2% of CD45+ cells) and the liver (from 5% to 10% of CD45+ cells) (Fig. 4 A-D). Ethanol feeding did not lead to a further increase in the systemic levels of activated neutrophils indicating the primary role of the AH-patient-derived gut microbiome in initiating the systemic inflammatory changes. Similarly to indigenous mouse microbiome (IMM) transplantation, mice with fecal
microbiome from normal healthy controls (no AUD) did not induce systemic inflammation marked by an increase in activated neutrophils in the spleen or the liver (Fig. 4 A-D).

Examination of the associated changes in the brain parenchyma mirrored systemic inflammatory changes demonstrating a significant increase in the number of infiltrated PMNs only in mice transplanted with the AH-fecal microbiome from an average of 48 neutrophils to an average of 383 in the PF-AH-FMT and 521 in the AF-AH-FMT per hemisphere (Fig. 4 E-G). Taken together, these data support the role of AH-microbiome in driving both peripheral inflammation and initiating a low grade inflammatory response in the brain. In tandem with the AH-FMT driven neutrophilia, were the dysbiotic microbiome transplantation increases in IL-17a from a concentration average of 2 mg/g of brain tissue to 9.6 mg/g in the PF and 9.8 mg/g in the AF AH-FMT mice, a significant chemokine/chemoattractant that drives neutrophil recruitment (Fig. 4 G). Commensurate with the general increase in inflammation in response to both ethanol and AH-FMT, the percentage of anti-inflammatory IL-10+ CD45+ cells were reduced in the brains from PF (21.7%) to AF (6.5%) AH-FMT recipients (PF:11.4%; AF: 2.5%) (Fig. 4 H).

**Regional astrocyte activation is induced by the pathogenic AH-FMT.**

Figure 5 shows an H&E-stained sagittal section of the mouse brain with the boxes indicating the specific regions examined: prefrontal cortex, hippocampus, and cerebellum. We did not see any changes in glial fibrillary acidic protein (GFAP) expression in the prefrontal cortex in response to our treatments (data not shown). In the hippocampus, the GFAP concentration in our control groups, PF (no fold change) and AF (1.1 fold change over PF), which both harbored the IMM, was unchanged (Fig. 6 C,E). However, when AF mice were transplanted with the microbiome from Patients with AH GFAP concentration was drastically increased (6.6 fold change over PF) (Fig. 6 C,E). Interestingly, in the cerebellum GFAP concentration was significantly increased in mice transplanted with the AH-microbiome regardless of ethanol consumption (PF:4.7;
AF: 4.8 fold change over PF) (Fig 6 D,F). In both the hippocampus and cerebellum, there was no significant change with the FMT from healthy controls (Fig. 6 G-J).

Interestingly, microglial activation was not detected in any CNS region, as assessed by IBA1 immunoreactivity or 3D reconstruction to examine changes in ramification (data not shown).

**The pathogenic microbiome of Patients with AH or ethanol consumption drives a neuronal ERSR**

In AH-FMT recipient mice, multiple regions of the CNS exhibited markers of an enhanced sub-acute ERSR, as immunohistochemical analyses demonstrated significant increases in KDEL, XBP1 and CHOP. In the prefrontal cortex, neurons across all layers displayed increased levels of GRP78 and GRP94 (SEKDEL) in response to AH-FMT (PF: 934; AF: 3266.7 fold change over PF) or ethanol consumption (1910.9 fold change over PF) independently (Fig. 7 A,B). The AF-IMM mice drove a higher average in SEKDEL expression than PF-AH-FMT but was not significantly different (Fig. 7 A,B). The AF-AH-FMT had the highest average (3266.7 fold change over PF) but the variance was too large between the AF and AF-AH-FMT leading to no significance between these groups in the prefrontal cortex (Fig. 7 A,B). Further assessment of the ERSR, indicated that the IMM, even with ethanol consumption, did not drive expression of XBP1 nor CHOP, but when AF mice had the pathogenic AH microbiome they began to express both XBP1 (56.3 fold over PF) and CHOP (759 Fold over PF) (Fig. 7 A,C,D). Further emphasizing the role of the microbiome, transplantation with nFMT did not drive these mice to express any ERSR protein (Fig. 7 E-J).

The initiation of the ERSR was also evident in the hippocampus after ethanol feeding (Fig. 8 A,B). Specific regions of the hippocampus, especially the dentate gyrus, presented significant increases in GRP78 and GRP94 in response to ethanol consumption regardless of microbiome perturbations (AF: 6.4; AF-AH-FMT: 13.3 fold
over PF) (Fig. 8 A,B). The markers of ERSR, XBP1 and CHOP, both were only elevated in the AF-AH-FMT (XBP1:7.2; CHOP:759 fold over PF) group while in the AF-IMM group there was no increase in these markers (Fig 8 A,C,D). These data indicate that alcohol consumption is sufficient to initiate the ERSR in the dentate, but the AH-FMT is the primary driver of XBP1 and CHOP expression. Interestingly, nFMT mice who consumed ethanol were protected from the ethanol driven expression of GRP78 and GRP94 (no fold change) (Fig. 8 E,G).

In the cerebellum, the Purkinje cells (PC), specifically labeled by PCP4, were uniquely affected. The AF in with both the IMM (9.3 fold over PF) and the AH-FMT (28.7 fold over PF) significantly increased levels of both GRP78 and GRP94 immunostaining (Fig. 9 A-C). Further assessment of the ERSR found that there was an increase in CHOP expression solely in the AF-AH-FMT group (6696 fold over PF) (Fig. 9 B,D). Notably, in the cerebellum there was no XBP1 expression. The eubiotic nFMT did not induce an increase in the ERSR, indicating the pathogenic nature of the AH microbiome is critical in inducing the ERSR in PCs (Fig. 9 F,H).

Immunostaining for cleaved Caspase-3 and Fluoro Jade C did not indicate increased neuronal death in this model (data not shown). Lack of neuronal death indicates the CHOP expression has not begun to initiate apoptosis.

Targeting the pathogenic dysbiosis with tributyrin differentially effects the sub-acute inflammation and ER stress induced by ethanol and/or AH-FMT in the various regions of the brain.

Targeting the loss of butyrate induced by chronic ethanol consumption and/or AH-FMT had differential effects on the three CNS areas examined (prefrontal cortex, hippocampus, and cerebellum). In the prefrontal cortex, there was no notable up regulation of GFAP (data not shown), and TB did not affect the activation of astrocytes in this region (Fig. 10 A,B). In contrast, in both the hippocampus (AF-AH-FMT+TB: 0.03
fold over PF) (Fig. 10 H,K) and cerebellum (AF-AH-FMT+TB: 2.1 fold over PF) (Fig. 10 O,R), TB did significantly reduce the expression of GFAP. TB was effective at reducing the ERSR activation in the various regions. In the prefrontal cortex, there was no significant reduction of ERSR related proteins (Fig. 10 C,D). Notably, all measures of ER stress were significantly reduced in the hippocampus by TB supplementation: SEKDEL (AF-AH-FMT+TB: 0.556 fold over PF), XBP1 (AF-AH-FMT+TB: 0.0317 fold over PF), and CHOP (AF-AH-FMT+TB: 153.6 fold over PF) (Fig. 10 I,J,L-N). In the cerebellum, TB did significantly reduce PC expression of GRP78 and GRP94 (AF-AH-FMT+TB: 0.606 fold over PF) (Fig. 10 P,S), as well as PC CHOP (AF-AH-FMT+TB: 1101.6 fold over PF) expression (Fig. 10 Q,T).

Discussion

The GM has become an increasingly important in our understanding of ethanol-induced pathology. Such pathology goes beyond the local environment in the intestines, where ethanol and its related dysbiosis leads to significant damage (Bishehsari et al., 2017). Our previous work demonstrated that mice that consume 5% ethanol exhibit signs of gut microbial dysbiosis after 5 weeks and by 8 weeks show dysbiosis similar to that seen in humans with AH (Bull-Otterson et al., 2013; Singhal et al., 2021). However, in these studies, delineating the ethanol vs microbiome driven pathology is impossible. Therefore, we developed a novel model that employed the oral transfer of dysbiotic microbiota from human Patients with AH by FMT along with a sub-acute ethanol feeding to understand the ethanol vs microbiome pathologies.

Our preclinical data demonstrate that a key component in the development of ethanol-induced gut-associated pathology is the gut microbial dysbiosis characterized by a significant loss of butyrate-producing bacteria (Bull-Otterson et al., 2013; Singhal et al., 2021). Interestingly the transplanted microbiome in the AH-FMT mice were also
characterized by a loss of butyrate producing bacteria, indicating that ethanol may have a specific effect on butyrate producers. Butyrate is known to mediate the maintenance of the gut barrier, to induce anti-microbial effects in macrophages (Schulthess et al., 2019), to regulate antibody production, and to support either Treg, Th1 or Th7 cell development, depending on the context of T cell activation (C. H. Kim, 2021; M. Kim, Qie, Park, & Kim, 2016; M. H. Kim, Kang, Park, Yanagisawa, & Kim, 2013; Park et al., 2015; Smith et al., 2013). An immediate loss of butyrogenic potential in the gut microbiota through AH-FMT alters hepatic immune responses and leads to increased hepatic and systemic (spleen) neutrophils. Consequently, AH is characterized by an increase in the infiltrating neutrophils into livers indicating this transplantation did aid in phenocopying the pathology (Jaeschke, 2002). The increase in splenic neutrophils that are recruited to the CNS appears to be mediated IL-17a (Fig. 4 H). Not only did the dysbiotic microbiota increase PMN infiltration to the CNS, but it also modified the resident immune cells as seen by the decreased number of IL-10-producing immune cells (Fig. 4 I, J). IL-10 is a critical immune suppressor, and the CNS has a limited capacity to produce it (Qin et al., 2008). Collectively, these data provide strong evidence that the AH-FMT primes and actively modulates the immune system both peripherally and in the CNS towards a proinflammatory state.

Studies on the brain structure of AUD patients have shown a reduction of volume in the prefrontal cortex, hippocampus, and cerebellum (Agartz et al., 1999; Andersen, 2004; X. Yang et al., 2016). Since the onset of this pathology is not well understood, we sought to determine if its development is related to dysbiotic microbiota as well as ethanol consumption. Strikingly, our data indicate that AH-FMT is a primary driver initiating astrocyte activation in both the hippocampus and cerebellum, since GFAP is increased in AH-FMT in these regions regardless of EtOH consumption. Interestingly, in the hippocampus the AF-AH-FMT astrocyte activation is unique to the hippocampus and
ethanol had little effect in the cerebellum. The fecal microbiota from healthy controls (nFMT) did not initiate astrocyte activation in any of the brain regions examined. Notably, the FMT from healthy controls with significantly greater microbial diversity and butyrate-producing bacteria were able to prevent ethanol-induced hippocampal astrocyte activation. These data not only emphasize the neuroinflammatory effects of the AH dysbiotic microbiota, but also implicate the therapeutic potential of a diverse healthy GM. These data also suggest that the cerebellum is specifically sensitive to the perturbations of the microbiome induced by chronic ethanol consumption.

The immune cell infiltration, IL-17a production, and astrocyte activation had a negative effect on neurons across various regions of the CNS. In the prefrontal cortex, neurons were sensitive to ethanol consumption paired with AH-FMT leading to expression of SEKDEL, XBP1 and CHOP suggesting that these neurons initiated an ERSR. Prolonged ERSR could lead to chronic dysregulation of proteostasis leading ultimately to apoptosis (Hu, Tian, Ding, & Yu, 2018). Damage to the neurons of the prefrontal cortex is known to modulate personality and could play a role in drug seeking behavior as well as impulsivity (Waszkiewicz et al., 2018). These behaviors are prevalent in patients addicted to ethanol (Jentsch & Taylor, 1999; Kalivas, 2009) and linking these behavioral changes to pathogenic alterations of the GM would provide a new therapeutic target in the treatment of these patients. Indeed, when targeting the loss of butyrate by TB supplementation, there was a reduction of SEKDEL, XBP1, and CHOP positive neurons in the hippocampus (Fig. 10 H,-N). Neurons of the dentate gyrus were affected by ethanol consumption and/or AH-FMT. These neurons, critical for the formation of memories and playing a role in depression (Vivar & van Praag, 2013), were affected by ethanol consumption and/or AH-FMT. Neurons within the dentate gyrus expressed SEKDEL in response to ethanol consumption with no additive effect when combined with AH-FMT. Expression of XBP1 or CHOP required the combination of
ethanol consumption with AH-FMT. Here again, nFMT surprisingly protected the neurons of the dentate from expressing ERSR markers, even in mice that consumed ethanol, pointing to a role of the microbiome in the treatment of ethanol-related neurological pathologies. Moreover, when targeting the pathological changes in the microbiome with TB, we found that astrocyte activation in the region was significantly reduced and neuronal SEKDEL and CHOP and Xbp1 expression in the dentate gyrus, indicating a reduction in the ERSR in these neurons.

Damage to cerebellar PCs results in ataxia (Hoxha et al., 2018), which is also a common behavior of patients addicted to ethanol, especially those going through withdrawal (Trevisan, Boutros, Petrakis, & Krystal, 1998). Animal studies on the effect of ethanol on neurocircuitry has been well established, showing acute ethanol can specifically affect the way PCs react to incoming signals (G. J. Zhang et al., 2017). These changes fail to describe the mechanism behind the cerebellar deficits seen in patient with AUD and only describe short term alcohol exposures. Chronic alcohol consumption in patients can lead cerebellar volume reductions as well as specifically a loss of PC density (Andersen, 2004; Baker et al., 1999). Here we provide evidence that the microbiome specifically affects the state of the PCs showing that simply transplanting the microbiome from patients leads to the expression of chaperon proteins (Fig. 9 A, B). When PCs express an increase in chaperon proteins and CHOP over a prolonged period, it can lead to astrocyte activation and eventual PC apoptosis (Y. Yang et al., 2018). Furthermore, the loss of PC density found in patients with AUD may follow the documented ERSR, particularly the expression of CHOP in the PC due to the exacerbated effects of the microbiome caused by ethanol consumption. Providing further evidence of the critical role of the microbiome, we targeted the functional loss of butyrate through oral TB supplementation which abrogated the ERSR in the PCs. Overall, we have identified a critical link between PC’s proteostasis network and gut microbiomial
dysbiosis marked by a loss of butyrate-producing bacteria and loss of the critical mediator butyrate, as well as provided evidence that targeting the microbiome is a critical therapeutic target in the treatment of patients with AUD that display ataxia. These data support the hypothesis that critical changes in the GM as a result of ethanol consumption induce neuropathology in the PCs and that this pathology, and presumably the resultant ataxia, can be at least partially abrogated by providing butyrate directly to the gut.

We provide evidence in this model of ethanol consumption that the microbiome plays a critical role in the development of neuropathologies. These initial data across multiple regions of the brain that are affected by these perturbations also demonstrate that butyrate homeostasis is critical for these effects on both astrocytes and the neurons. While we did not see microglial activation or frank neuronal death, we suggest that increasing the temporal extent of ethanol consumption would lead to both of these outcomes. Finally, we have identified several therapeutic strategies that may provide effective treatment of CNS neuroinflammation. The identification of the loss of butyrate-producing gut bacteria led to TB treatment to restore butyrate. Moreover, the prevention of ethanol-induced dysbiosis with a FMT from a healthy control prevented the pathologies seen with ethanol consumption alone. Of particular importance was the reduced activation of the ERSR allowing for normal functioning of neurons. The CNS pathogenic effects of alcohol-induced dysbiotic GM are evident and when further combined with ethanol consumption are exacerbated. Current data provide further evidence that microbiota have a critical role in both peripheral and CNS pathogenesis. As the development of therapies for ethanol-related injury continues, one of the focal points must be the gut microbiome.

Acknowledgements
A thanks to: The dash consortia (Crabb et al., 2016) for fecal donation from Patients with AH; Baylor University for microbiome sequencing and analyzes by Smita Ghare, Ph.D. and Richa Singhal; Ph.D. Manicka Vadhanam Ph.D. for aiding in the development of the FMT protocol; Scott Myers, Ph.D. for training and aiding in staining ERSR related markers and hippocampal imaging. Paula Chilton for training and aiding in FACs staining; The metabolomics core for processing and running fecal sample GCMS for SCFAs.
FMT animal model

- C57BL/6 Male Mice
- Iso-caloric control: Pair-Fed (PF)

Figure 1: Experimental design (Chapter 2)
Figure 2: Ethanol induces dysbiosis characterized by the loss of butyrate-producing bacteria

Plot microbiome study with n=13 Alcoholic hepatitis (AH) patients and n=8 healthy subjects (normal). Alpha Diversity for normal human subjects and alcoholic hepatitis subjects. Diversity measures A) Chao1 Index and B) Shannon Index. Whisker plots for A and B show 25th, 75th percentile, mean with the min and max. Relative Abundance analysis for two largest butyrate-producing families in phylum Firmicutes – C) Lachnospiraceae and D) Ruminococcaceae. E) 37 different butyrate-producing genera belonging to Firmicutes families. Red color signifies that genus is present in low abundance or absent and green color signifies that genus is highly abundant. F) the fecal butyrate levels were analyzed using GC-MS. Graphs are represented as mean ± SEM, n=8 or 13
### Table 1: Demographic and clinical characteristics between AH subjects and matched controls

<table>
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<th>Normal (n=9)</th>
<th>AH (n=13)</th>
<th>p value</th>
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<td><strong>Demographics</strong></td>
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<td>Age (years)</td>
<td>44.3 ± 14.9</td>
<td>46.6 ± 11.6</td>
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<td><strong>Sex</strong></td>
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<tr>
<td>Male</td>
<td>4 (44.5%)</td>
<td>8 (61.5%)</td>
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<tr>
<td>Female</td>
<td>5 (55.6%)</td>
<td>5 (38.4%)</td>
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<td><strong>Clinical characteristics</strong></td>
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<tr>
<td>MDF</td>
<td>n.d.</td>
<td>55.9 ± 29.9</td>
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<td><strong>Liver Function Tests</strong></td>
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<td>AST (SGOT)</td>
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<td>ALT (SGPT)</td>
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<td>Total protein</td>
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<td>CK18M65</td>
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AST: aspartate aminotransferase; ALT: alanine aminotransferase; CK: cytokeratin; n.d.: not determined; MCP-1: monocyte chemotactic protein-1; MDF: Maddrey discriminant function; MELD: model of end-stage liver disease; SGOT: serum glutamic oxaloacetic transaminase; SGPT: serum glutamic pyruvic transaminase; TNFα: tumor necrosis factor α
Figure 3: Successfully transplanted AH-FMT phenocopies AH dysbiosis.

Diversity analysis: A) Alpha diversity measure Shannon Index evaluating species abundance and evenness. Whisker plots for A and B show 25th, 75th percentile, mean with the min and max; B&C) Beta Diversity PCA plots comparing PF, PF+N-FMT, PF+AH-FMT groups and AF, AF+N-FMT, AF+AH-FMT groups representing unweighted Unifrac distances; E-H) Relative abundance analysis of two largest butyrate-producing families – Lachnospiraceae (E,F) and Ruminococcaceae (G,H) Graphs represented as mean ± SEM. statistical analysis is shown as Kruskal Wallis analysis p value
significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = **** with an n=8-24
Figure 4: AH-FMT elicits immune response driving neutrophil infiltration and cytokine expression

A, B) Representative FACS plots from the Spleens and liver from animals treated with FMT and/or AF defining PMNs as CD45+/CD11b+/Ly6G+. C-D) Quantification of FACS plots from A (D) Quantification B (E) calculation of total number of PMNs. FACS plots
from the brains of animals treated with FMT and/or AF defining PMNs as CD45+/CD11b+/Ly6G+ (F) Quantification of FACS plots in E calculation of total number of PMNs. (G) IF of neutrophils in the parenchyma (H) Brain concentration of IL-17a ELISA across treatment FMT+/− AF (I) Representative FACS plots from the brains of animals treated with FMT and/or AF analyzing CD45+/IL-10+ immune cells (J) Quantification of percentage positive H. Graphs represented as mean ± SD. n=3-15. Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
Figure 5: Sagittal section of mouse brain stained with H&E showing regions studied

Whole brain from an untreated mouse. To show various regions studied
Figure 6: Regional differences in astrocyte activation in response to AH-FMT.
A) GFAP stain sagittal section of murine brain showing regional expression of GFAP (FITC) and studied regions
B) Whole cerebellum with white box indicating size of region
analyzed (C) representative images from the Dentate Gyrus of the Hippocampus in mice treated with FMT from an AH patient and/or AF stained with GFAP(FITC) (D) representative images from the cerebellum in mice treated with FMT from an AH patient and/or AF (E) Quantification of GFAP in dentate gyrus in mice treated with FMT and/or AF by area positive analysis and normalizing respective experiments to their PF (F) Quantification of GFAP in cerebellum in mice treated with FMT and/or AF by area positive analysis and normalizing respective experiments to their PF (G) Representative images from animals treated with FMT from a healthy control of the dentate gyrus by area positive analysis and normalizing respective experiments to their PF (H) Representative images from animals treated with FMT from a healthy control of the cerebellum by area positive analysis and normalizing respective experiments to their PF.

Graphs represented by mean ± SD. n=5-13. Statistics: Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
Figure 7: Neuronal ERSR is driven by AF-AH-FMT in the prefrontal cortex

A) Representative images from the prefrontal cortex of mice treated with FMT from Patients with AH and/or consuming AF stained with NeuN, KDEL, XBP1 and CHOP. (B) Quantification of KDEL/NeuN in A by area positive analysis and normalizing respective experiments to their PF (C) Quantification of XBP1/NeuN in A by area positive analysis and normalizing respective experiments to their PF (D) Quantification of CHOP/NeuN in A (E) Representative images from animals who received FMT from a healthy control
stained with KDEL and NeuN (F) Representative images from animals who received FMT from a healthy control stained with CHOP, XBP1 and NeuN (H) Quantification of KDEL/NeuN in E by area positive analysis and normalizing respective experiments to their PF (I) Quantification of XBP1/NeuN in F by area positive analysis and normalizing respective experiments to their PF (J) Quantification of CHOP/NeuN in F. Graphs represented by mean ± SD. n=5-13. Statistics as Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
Figure 8: Neuronal ERSR is driven by AF-AH-FMT in the prefrontal cortex

A) Representative images from the dentate gyrus of hippocampus of mice treated with FMT from Patients with AH and/or consuming AF stained with NeuN, KDEL, XBP1 and CHOP. (B) Quantification of KDEL/NeuN in A by area positive analysis and normalizing respective experiments to their PF (C) Quantification of XBP1/NeuN in A by area positive analysis and normalizing respective experiments to their PF (D) Quantification of CHOP/NeuN in A (E) Representative images from animals who received FMT from a
healthy control stained with KDEL and NeuN (F) Representative images from animals who received FMT from a healthy control stained with CHOP, XBP1 and NeuN (H) Quantification of KDEL/NeuN in E by area positive analysis and normalizing respective experiments to their PF (I) Quantification of XBP1/NeuN in F by area positive analysis and normalizing respective experiments to their PF (J) Quantification of CHOP/NeuN in F by area positive analysis and normalizing respective experiments to their PF. Graphs represented by mean ± SD. n=5-13. Statistics: Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
Figure 9: The Purkinje Cells specifically develop ERSR in the brains of AF-AH-FMT mice

A) Cerebellum stained with NeuN and PCP4 showing PCP4 as a better marker of Purkinje Cells (B) Representative images from the cerebellum of mice treated with FMT
from Patients with AH and/or consuming AF stained with PCP4, KDEL and CHOP. (C) Quantification of KDEL/PCP4 in B by area positive analysis and normalizing respective experiments to their PF (D) Quantification of CHOP/PCP4 in B by area positive analysis and normalizing respective experiments to their PF (E) Representative images from animals who received FMT from a healthy control stained with KDEL and PCP4 (F) Representative images from animals who received FMT from a healthy control stained with CHOP and PCP4 (G) Quantification of KDEL/PCP4 in E by area positive analysis and normalizing respective experiments to their PF (H) Quantification of CHOP/PCP4 in F by area positive analysis and normalizing respective experiments to their PF. Graphs represented by mean ± SD. n=5-13. Statistics: Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
Figure 10: TB significantly reduces dysbiosis driven neuronal ERSR in hippocampus and cerebellum

A) Representative images of GFAP stained in the prefrontal cortex from AF-AH-FMT mice treated with TB (B) quantification of GFAP from A by area positive analysis and normalizing respective experiments to their PF (C) Representative images of KDEL/NeuN stained in the prefrontal cortex (D) quantification of KDEL/NeuN from C by area positive analysis and normalizing respective experiments to their PF (E)

Representative images of XBP1, CHOP and NeuN stained in the prefrontal cortex (F)
Quantification of XBP1/NeuN from E by area positive analysis and normalizing respective experiments to their PF (G) Quantification of CHOP/NeuN from E by area positive analysis and normalizing respective experiments to their PF (H) Representative images of GFAP stained in the dentate gyrus of the hippocampus from AF-AH-FMT mice treated with TB (I) Representative images of KDEL and NeuN stained in the dentate gyrus of the hippocampus from AF-AH-FMT mice treated with TB (J) Representative images of XBP1, CHOP and NeuN stained in the dentate gyrus of the hippocampus from AF-AH-FMT mice treated with TB (K) Quantification of KDEL/NeuN from I by area positive analysis and normalizing respective experiments to their PF (L) Quantification of XBP1 or CHOP/NeuN from J by area positive analysis and normalizing respective experiments to their PF (O) Representative images of GFAP stained in the cerebellum from AF-AH-FMT mice treated with TB (P) Representative images of KDEL/PCP4 stained in the cerebellum from AF-AH-FMT mice treated with TB (Q) Representative images of CHOP and PCP4 stained in the cerebellum from AF-AH-FMT mice treated with TB (R) Quantification of GFAP from O by area positive analysis and normalizing respective experiments to their PF (S) Quantification of KDEL/PCP4 from P (T) Quantification of CHOP/PCP4 from Q by area positive analysis and normalizing respective experiments to their PF. Graphs represented by mean ± SD. n=5-13.

Statistics performed as Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
CHAPTER 3:
TARGETING THE FUNCTIONAL LOSS OF BUTYRATE IN GUT MICROBIAL DISBIOSIS PREVENTS PROGRESSION OF ALZHEIMER’S DISEASE

Introduction

Alzheimer’s Disease (AD) is a progressive neurodegenerative disease that affects multiple neurologic functions, including learning, memory retention, language usage and neuromuscular coordination. The progression of the disease is long-term, often initiating decades prior to symptom development (Braak, Thal, Ghebremedhin, & Del Tredici, 2011; Gordon et al., 2018; Reiman et al., 2012). After presentation, symptoms progress from mild learning, memory, and language problems to deficits in basic bodily functions, including speaking, swallowing, and walking (“2020 Alzheimer’s disease facts and figures,” 2020). A large body of evidence points at increased oxidative stress in the AD brain, as measured by accumulation of reactive aldehydes, such as 4-hydroxynonenal (4-HNE) and 2-propenal (acrolein), especially in the hippocampus, amygdala, and frontal cortex (Lovell, Ehmann, Butler, & Markesbery, 1995; Lovell, Xie, & Markesbery, 2001).

The most common histopathologic hallmarks of AD are: (1) the accumulation of foci in the CNS containing the $\gamma$-secretase-cleaved peptides from amyloid precursor protein (APP) or amyloid-$\beta$ (A$\beta$) peptides and (2) the appearance of neurofibrillary tangles (NFT) consisting of hyper-phosphorylated, denatured forms of the microtubule-associated protein tau. Polymorphisms of APP (APP$^{Swe}$)(Mullan et al., 1992), tau (tau$^{P301L}$)(Bugiani et al., 1999), and the $\gamma$-secretase protease, presenilin-1 (PS$^{1M146V}$)
Alzheimer’s Disease Collaborative, 1995) are associated with early-onset AD (Oddo, Caccamo, Shepherd, et al., 2003). The 3xTg mouse model transgenically expresses all three of these AD-associated forms of human proteins in the brain, which leads to the development of progressive neuropathology that can be detected as early as 2 months, with extracellular Aβ plaques by 4 months of age (Belfiore et al., 2019; Oddo, Caccamo, Kitazawa, Tseng, & LaFerla, 2003) followed by NFT detection near 12 months (Oddo, Caccamo, Shepherd, et al., 2003). That neuropathology is accompanied by cognitive and behavioral changes as early as 6 months (Gimenez-Llort et al., 2007; Sterniczuk, Antle, Laferla, & Dyck, 2010). Associated with the Aβ peptides and neuronal tangles is oxidative stress (Young & Franklin, 2019). The development of oxidative stress is observed in both human pathogenesis and animal models of AD, and precedes behavioral changes in those animals (Persson, Popescu, & Cedazo-Minguez, 2014). Oxidative stress is an early marker of AD known to increase Aβ production and further the development and accumulation of Aβ plaques (Paola et al., 2000).

The gut microbiome (GM) is a vast and diverse committee of organisms that have a symbiotic relationship with the host (Malys, Campbell, & Malys, 2015). The GM helps the host by aiding in digestion, maturation of the immune system and providing short chain fatty acids (Valdes, Walter, Segal, & Spector, 2018). However, when the bacterial communities in the GM are disrupted by loss of diversity, increase in pathobionts, and/or loss of beneficial bacteria, the positive functions of the GM can be interrupted (dysbiosis) and lead to inflammation (Al Bander, Nitert, Mousa, & Naderpoor, 2020). Gut microbial dysbiosis is also hypothesized to have direct effects on CNS oxidative stress (Bonfili et al., 2017; Jones, Mercante, & Neish, 2012). The GM has been linked to several CNS diseases such as depression and Parkinson’s disease, and AD is now also emerging as a gut related disease (Moustafa et al., 2021; Peirce & Alvina, 2019; Vogt et al., 2017).
Recent studies have described AD-associated alterations in the GM and GM-derived metabolites. The GMs of patients with AD show decreased biodiversity distinct from age- and sex-matched individuals (Vogt et al., 2017). GM dysbiosis positively correlates with AD progression, as characterized by increased endotoxemia, increased pro-inflammatory cytokine release, and decreased levels of anti-inflammatory short-chain fatty acids (SCFA) and cytokines, butyrate and IL-10, respectively (Marizzoni et al., 2020). A dysbiotic GM profile, with higher pro-inflammatory taxa *Escherichia/Shigella* coupled with lower anti-inflammatory *E. rectale*, correlated with increased amyloidosis in a cohort with demonstrated cognitive impairment (Cattaneo et al., 2017). Other studies in a Japanese population showed that decreases in the family *Bacteroides* in the GM were associated with dementia (Saji et al., 2019) while higher fecal lactic acid levels were associated with a lower risk of dementia. In addition, higher fecal ammonia levels were possibly associated with higher dementia (Saji et al., 2020).

Age-dependent progression of GM dysbiosis was noted in mice transgenic for human APP and PS1 genes (APP/PS1 mice) when compared to wild-type mice (Harach et al., 2017). Germ-free APP/PS1 transgenic mice had decreased Aβ pathology and transfer of GM from conventionally raised APP/PS1 mice restored the Aβ pathology (Harach et al., 2017). Bonfili and colleagues demonstrated that modulation of the GM by providing a probiotic formulation in the drinking water to 3xTg mice beginning at 8-weeks delayed disease progression and eliminated many of the AD-related cognitive and behavioral symptoms (Bonfili et al., 2017; Bonfili et al., 2018; Bonfili et al., 2020). In these studies, multiple cellular and molecular mechanisms affected by the presence of the transgenes were abrogated by the manipulation of the GM. These mechanisms include gut peptide hormone levels in the plasma, neuronal proteolysis, neuronal and peripheral glucose homeostasis, gut levels of SCFA, and increased antioxidant effects in the brain through increased SIRT1 expression (Bonfili et al., 2017; Bonfili et al., 2018;
Bonfili et al., 2020). In the current study, 3xTg mice were used to test the hypothesis that oral supplementation with a butyrate prodrug, tributyrin, will help protect against AD pathology and cognitive/behavioral changes through the gut-liver-brain axis.

**Methods:**

**Animals.**

The triple transgenic mouse model of early-onset AD (referred to as 3xTg)(n=5,6), B6;129-\(P_{sen}1^{tm1Mpm}\) Tg (APPSwe, tauP301L)1Lfa/J mice developed at the University of California by Frank LaFerla (Oddo, Caccamo, Shepherd, et al., 2003) were purchased from Mutant Mouse Resource and Research Center and bred at the University of Louisville. Wild type B6;129 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used as controls.

**Experimental Design.**

6-month-old female 3xTg mice were separated into two groups (n=5,6). One group was treated twice weekly with 5 mg/kg tributyrin oral administration until behavioral tests were performed, and one group was left untreated. At 16 months, these mice along with 10-month-old 3xTg, 2-month-old 3xTg and 2-month-old non-transgenic wild type mice were subjected to the behavioral tests described below. At 18-months, 3xTg groups were anesthetized and blood from the inferior vena cava was collected for serum samples. After perfusion with ice cold phosphate buffered saline (PBS), spleen, liver, cecal contents, fecal pellets and intestine were collected and tissue sites clamped off to allow for additional perfusion with 20 ml 4% formaldehyde (freshly prepared 4% wt/vol from paraformaldehyde in PBS, pH 7.2). Perfused brains were then harvested, dissected into hemispheres, and fixed overnight in 4% formaldehyde solution.
**Metagenomic Analysis**

Genomic bacterial DNA was extracted from fecal samples using MagAttract PowerSoil Kit (Qiagen). 16S rRNA gene sequencing methods were adapted from the NIH-Human Microbiome Project (Human Microbiome Project, 2012a, 2012b). The 16S rDNA V4 region is amplified by PCR using primers (GGACTACHVGGGTWTCTAAT and GTGCCAGCMGCGCGGTAA) that contain adapters for MiSeq sequencing and dual-index barcodes so that the PCR products may be pooled and sequenced directly targeting minimum 10,000 reads per sample (Caporaso et al., 2012; Caporaso et al., 2011). Amplicon libraries were purified using SPRI beads on a Biomek liquid handler. DNA was quantitated by Quant-iT PicoGreen assay, normalized, pooled and followed by large-scale parallel sequencing on the Illumina MiSeq platform using the v2 SBS chemistry. An operational taxonomic units (OTUs) table was generated by taxonomic binning via the 1.91 version of Quantitative Insights into the Microbial Ecology (QIIME) (Caporaso et al., 2010) and its various components (Caporaso et al., 2010; DeSantis et al., 2006) using default settings unless otherwise noted. Closed reference OTU picking was performed using OTUs with 97% similarity to the clustered 13_8 release of the Greengenes reference database packaged with QIIME (McDonald et al., 2012). To maximize the sensitivity of detecting microbial taxa, the sequence reads after quality filtering were inputed into the RDP Classifier (Q. Wang, Garrity, Tiedje, & Cole, 2007) to generate a table of taxonomic abundances in each sample. Default settings were used with the RDP Classifier except the confidence cutoff which was set at 0.5 and the format which was set as fixrank. Further QIIME 1.91 pipeline generated output for alpha-diversity and beta-diversity and provide taxonomic summaries that are leveraged for all subsequent analyses.
**PICRUSt analysis**

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013) was used for predictive analysis of the butyrate synthesis pathway. This computational approach uses 16S rRNA marker gene data in combination with a table of gene copy numbers of enzyme families present within each sequenced archaeal and bacterial taxonomic group in the IMG (Integrated Microbial Genomes) database. The output consists of the counts of coding genes from the Kyoto Encyclopedia of Genes and Genomes (KEGG). PICRUSt was also used to generate output showing the contribution of each OTU to each KEGG gene. Default settings of PICRUSt were used except during the generation of OTU contributions to genes where only butyrate synthesis coding genes were retrieved.

**Statistical Analysis**

All statistical tests were performed using nonparametric tests - Mann U Whitney test, Kruskal Wallis test with corrections (Dunn’s multiple comparisons adjusted p-value or Bayesian q-value) (Carvajal-Rodriguez & de Una-Alvarez, 2011) and Spearman correlations.

**Behavioral and neural functional assessments.**

Animals were handled for at least 3 days prior to testing to acclimate them to the experimental conditions and tested a month before terminating the experiment. Groups of 3xTg mice were randomized to blind the testing investigator of their treatment or age group. The *Novel object recognition test* (NORT) measures the development and retention of recognition memory using the instinctual tendency of rodents to explore novel objects compared to familiar objects (Antunes & Biala, 2012). After a training period in an open field containing two identical objects, mice were removed from the
area for 24 h and then reintroduced to the area containing a familiar object and a
dissimilar novel object. The amount of time each mouse spends exploring each object in
the field is recorded, measured, and compared. Results are expressed as
Discrimination Index \((\text{Time}_{\text{novel}} - \text{Time}_{\text{familiar}})/\text{Time}_{\text{novel}} + \text{Time}_{\text{familiar}})\), and Recognition Index
\((\text{Time}_{\text{novel}}/\text{Time}_{\text{novel}} + \text{Time}_{\text{familiar}})\). With each of these indexes, the lower the score is
indicative of greater impairment.

The Y-maze test is a spontaneous alternation test used to assess spatial working short-
term memory (Kraeuter, Guest, & Sarnyai, 2019). Like the NORT, the Y-maze test uses
the rodent’s natural preference to investigate new areas to measure short-term memory.
Mice were placed in the center of a Y-maze with either three or two arms opened, and
their movements were recorded for 8 minutes. The Discrimination Index (discrimination
between the novel and familiar objects) with 3 opened arms and the percentage of
alternation observed with 2 opened arms were used as markers for spatial memory and
behavioral alteration, respectively. The lower the scores, the greater the functional
deficits.

Grip Test was used to assess neuromuscular function (Mettlach et al., 2014). Mice were
lifted by the tail to the height where the front paws were the same height as a horizontal
bar and then encouraged to grip the bar. Once symmetric, tight grips of both paws were
ensured, the mouse was slowly and gently pulled away until its grasp is broken. The
force at which the grip was lost was recorded 3-5 times to determine best performance
which is then noted as grip force. Grip strength was calculated as grip force per body
weight in grams. Both measurements are directly proportional to neuromuscular
function.

The Rotarod Test was used to assess motor coordination and balance (Shiotsuki et al.,
2010). With no training period, the latency time (amount of time each mouse remained
on the rotating rod before falling) was measured three times separated by 15-minute intervals. The rotation speed was increased from 4 to 40 rpm up to 300 seconds.

**Brain sectioning and immunofluorescence.**

After embedding in paraffin, sections where cut at 5 μm thickness, floated in water, adhered to slides, and then incubated at 60°C for 48 hrs. Slides were treated with Trilogy reagent (Cell Marque) to remove paraffin, rehydrate the tissues, and retrieve antigens. Slides were processed as previously described (Avila et al., 2017). Briefly, immunostaining was performed for detecting the presence of activated microglia (Iba-1; Wako; Richmond, VA), amyloid-β (Cell signaling, Danvers, MA), AT-8 (ThermoFisher), 4-HNE (Alpha Diagnostics, Burlington, NC), acrolein (Cell Sciences, Newburyport, MA), and neurons (NeuN; ABN90P; Millipore; Temecula, CA). See table 2 for more information on antibodies. DAB staining was performed using VisUCyte™ Cell and Tissue Staining Kits (R&D Systems) and counterstained with hematoxylin. Serial sections (n=3-5) were used to determine an average value for each animal. Values were only removed for damaged or folded tissues that prohibited accurate analysis. For each antibody, an isotype and no primary were used as controls. See data table 2 for antibody information.

**Results**

**Age-dependent structural and functional changes in the gut microbiota of 3xTg mice**

To evaluate age-dependent shifts in the gut microbiome of 3xTg transgenic mice, 16S rDNA sequencing of cecal contents was performed in mice aged 2 months, 10-12 months, and 18 months. A total of 380,989 mapped reads (mean ± SD: 14,111 ± 9,804) were obtained from the 28 mice from all groups that were then clustered into OTUs at
97% similarity. The final OTU dataset consisted of 485 OTUs classified into 81 genera, 36 families, 24 orders, 9 classes, and 7 phyla.

An increase in age is associated with a decrease in gut microbial diversity. The compositional diversity of the gut microbiome was assessed using both alpha and beta diversity measures. Compared to 2-month-old animals, mice 10-12 months of age exhibited a 50% reduction in sample richness as measured by observed OTU and Chao-1 (p<0.001; Fig. 11 A, B), both measures of alpha diversity. These reductions persisted at 18 months of age (p<0.05). Sample evenness, another component of alpha diversity, was also assessed using the Shannon Diversity Index (Fig. 11 C). Although reductions in sample evenness were evident in 10-12- and 18-month-old animals, only the latter was significantly different from 2-month-old mice. These data indicate that alpha diversity of the cecal microbiome significantly decreases with advancing age in 3xTg mice.

Beta diversity, a measure of inter-sample diversity, was assessed using the weighted Bray Curtis distance metric and visualized by principal coordinates analysis (PCoA). PCoA plots demonstrate distinct clustering of the cecal microbiota according to age groups that were significantly different (p<0.05). In particular, mice 10-12 months and 18 months of age each segregate from those in the 2-month age group (Fig. 11 D), suggesting a change in the overarching composition of the gut microbiome within the first 10-12 months in 3xTg mice and then it largely stabilizes.

An increase in age is associated with changes in gut microbiome composition (dysbiosis).

Seven phyla were detected in the cecal contents of our experimental mice (Fig. 14 A). Among these, Firmicutes and Bacteroidota, the 2 major bacterial phyla, showed a significant age-dependent change in their relative abundance. Specifically, the relative abundance of Firmicutes decreased by 50% in 10–12-month-old animals as compared
to mice 2 months of age (Fig.11 E). In contrast, the relative abundance of Bacteroidota increased by nearly the same amount over the same time period (Fig.11 F). Consistent with measures of microbial diversity, both of these shifts were maintained in animals 18 months of age (Fig.11 E, F). Importantly, the Firmicutes to Bacteroidota (F/B) ratio, a known marker for gut dysbiosis (Stojanov et al., 2020), was significantly decreased in both 10-12- and 18-months old mice as compared to those 2 months of age (Fig.11 G).

Age-associated gut dysbiosis is marked by a decrease in butyrate-producing bacteria. Preclinical and clinical studies suggest a role of SCFA produced by the gut microbiome in ameliorating AD pathology (Ho et al., 2018; Marizzoni et al., 2020; Silva, Bernardi, & Frozza, 2020; L. Zhang et al., 2017). Since we observed an age-dependent decrease in phylum Firmicutes, which harbors the majority of butyrate-producing microbes, we further examined the effect of age on the relative abundance of butyrate-producing bacteria in 3xTg mice. Based on the catalog of butyrate-producing bacteria created by Singhal et al (Singhal et al., 2021), five butyrate-producing families belonging to the Firmicutes phylum were identified. As with the analysis of Firmicutes as a whole, there was a significant decrease in total butyrate-producing families in 10-12 month and 18-month-old mice as compared to mice 2 months of age (Fig. 11 H). Specifically, data demonstrate that advanced age is associated with a significant decrease in the Ruminococcaceae family, one of the largest butyrate-producing bacterial families (Fig.11I) and a consequent reduction in cecal butyrate levels (Fig. 11 J).

Oral administration of TB mitigates the age-associated structural and functional changes in the gut microbiota of 3xTg mice

Based on the observed age-dependent loss of key butyrate-producing microbes and the accompanying decrease in cecal butyrate levels, the therapeutic effects of oral administration of TB were examined. Beginning at 6 months of age, 3xTg mice demonstrate Aβ protein deposition and initiation of AD phenotypes (Javonillo et al.,
Thus, oral administration of TB was initiated in 6-month-old mice and continued until the age of 16 months. Cecal samples were analyzed to assess the long-term therapeutic effects of oral TB supplementation in rectifying age-associated gut dysbiosis and attendant loss of butyrate-producing bacteria.

Alpha diversity analysis revealed that mice receiving TB had a higher degree of microbial evenness as measured by the Shannon Diversity Index (Fig. 13 A). Further, richness of the microbiome, as assessed by Chao-1 index, also trended to be higher in TB-treated mice (Fig. 13 B). Consistent with alpha diversity measures, weighted Bray-Curtis assessment of beta diversity indicated distinct compositional differences in animals undergoing TB treatment. Interestingly, TB-administered mice demonstrated less dispersion in their clustering as visualized by PCoA (Fig. 13 C) and appeared more similar to the 2-month age group, particularly along axis PC1, suggesting TB administration helps maintain a gut microbiome composition similar to that of young mice (Fig. 14 C).

Further, taxonomy-based analysis demonstrated TB-induced changes in the overall distribution of microbial phyla (Fig. 14 C). Specifically, the relative abundance of phylum *Firmicutes* was nearly 2-fold higher in TB-treated mice (Fig. 13 D). Although there was no significant change in phylum *Bacteroidota*, the F/B ratio was significantly greater in mice receiving TB (Fig. 13 E,F). These data suggest that oral TB reversed changes in gut microbiome diversity and dysbiosis that occurred in 18-month old 3xTg mice.

TB attenuates an age-associated loss in butyrate-producing gut bacterial families. Oral TB treatment showed that the relative abundance of total butyrate-producing bacteria was maintained at a significantly higher level in comparison to untreated mice (Fig. 13 G). Importantly, examination of the butyrate-producing bacteria demonstrated that oral
TB led to a significant enrichment in the major butyrate-producing family *Lachnospiraceae*, with only a marginal effect on *Ruminococcaceae* (Fig. 13 H,I). Further, commensurate with oral TB supplementation, cecal butyrate levels were significantly increased (Fig. 13 J)

**Predicted functional profiles of butyrate synthesizing genes affected by age and TB.**

Putative determination of butyrate synthesizing genes was achieved from PICRUSt2 output using in-house updated butyrate genes inventory. This predictive analysis showed that the gut microbiome of 10-12-month and 18-month-old animals had a significant reduction in the abundance of butyrate synthesizing genes associated with the four butyrate synthesizing pathways: acetyl-CoA, lysine, 4-aminobutyrate, and glutamate (Fig. 12). Further, the data suggest that among these four pathways, bacterial genes involved in the acetyl-CoA butyrate synthesizing pathway were most affected by the increase in age. Notably, oral TB supplementation significantly prevented this age associated decrease in butyrate synthesizing genes (Fig. 12). Moreover, the putative tributyrin esterase (estA) gene that hydrolyzes TB to generate butyrate was also predicted to be significantly reduced in these older age mice as compared to mice 2 months of age, indicating that aging potentially decreases the capacity of the gut microbiome to derive butyrate from dietary TB (Fig. 12). Collectively, these results demonstrate that a significant component of age-associated dysbiosis involves a decrease in butyrate-producing bacteria which can be treated by TB supplementation in 3xTg transgenic mice.

**TB reduces inflammation and oxidative stress in the hippocampus of 3xTg mice**

An increase in astrocyte and microglial activation, demonstrated by increased GFAP and IBA-1 staining, respectively, coincided with the age-associated development of AD-like pathology (P-Tau and Aβ) (Fig. 14 A,D; Fig. 16 C,D). Notably, TB supplementation led to a marked decrease in ionized calcium-binding adapter molecule
1 (IBA-1) expression from 4,563.8 μm at 18 months and 225.4 μm with TB supplementation, indicating reduced microglial activation, despite no reduction in the Aβ plaque burden (Fig. 15 A,B; Fig. 15 A,B). TB supplementation did not change Glial fibrillary acidic protein (GFAP) immunostaining, where GFAP had 96,520.5 μm and with 18 month with TB had 52,417.83 μm indicating no effect on the astrocyte activation (Fig. 15 C,D). However, increased GFAP has been linked to aging rather than as a correlative marker of AD pathology (Bronzuoli et al., 2019). These inflammatory events were localized in the subiculum, which is the region in which the most Aβ plaques and NFT developed. No microglia or astrocyte activation was observed in other regions of the hippocampus (data not shown).

Toxic accumulation of Aβ induces reactive oxygen species (ROS) formation, protein oxidation, and lipid peroxidation resulting in oxidative stress (Butterfield, Griffin, Munch, & Pasinetti, 2002). AD pathology in both human and mouse models including the 3xTg is characterized by oxidative stress which can lead to neuronal damage and death (Butterfield, 2002; Butterfield et al., 2002). Notably, both 4-HNE and acrolein adducts increased with age and were significantly present at 18 months puncta counts reaching 124.66 (4-HNE) and 131.8 (Acrolein) and these adducts were observed within the molecular layer and the stratum lucidum of the hippocampus, regions that were devoid of Aβ plaques and NFT (Fig. 16 C-F). Supplementation with TB markedly attenuated the accumulation of both 4-HNE and acrolein adducts in 18-month-old animals reducing to an average of 36.4 and 41.2 (punta counts) respectively (Fig. 15 C-F).

**Tributyrin supplementation does not abrogate Aβ but does prevent hyperphosphorylation of the tau**

As previously reported (Oddo, Caccamo, Shepherd, et al., 2003), 3xTg mice progressively develop AD-related accumulation of both Aβ plaques and NFTs in an age-related fashion, predominately in the most inferior component of the hippocampus, the
subiculum (Fig. 15 A,C), which has been linked to spatial memory and working memory (O'Mara, 2005; Riegert et al., 2004). TB supplementation did not affect Aβ plaque formation observed at 18 months, in which 18 months had an average of 135,035 μm positive area and 18 months+TB 112,549 μm (Fig. 16 A,B). Interestingly, staining with the P-Tau markers D2Z4G and AT-8 showed expected age-associated increases, but TB did not significantly affect those P-Tau levels (Fig. 16 D,E). The pathological confirmation of tau requires hyperphosphorylation, which is demonstrated via colocalization of D2Z4G and AT-8 (Fig. 17 A). Importantly, oral administration of TB prevented hyperphosphorylation of tau denoted by the area positive of the colocalized staining was reduced from 1,888.77 μm to 17.5 μm in the TB supplemented animals (Fig. 16 F).

**TB prevents age-dependent decreases of histone H3-K9/K14-Ac in the hippocampus**

A biological function of butyrate is inhibition of histone deacetylases (HDACs) affecting histone acetylation status (Davie, 2003). Notably, the total H3-K9/K14-Ac declined in the hippocampus with age in 3xTg brains, where the 2month 3xTg had an average 220,315 μm of positive area which was decreased to 137,144 μm by 18 months (Fig 18 A-C). These age-associated histone acetylation changes were detected throughout the hippocampus. Specifically, dentate gyrus neurons, which are critical for memory formation (Vivar & van Praag, 2013), had intense H3-K9/K14-Ac staining at 2 months that trended lower at 12 months and was significantly decreased at 18 months (Fig. 18 A-C). Importantly, the H3-K9/K14-Ac status of dentate gyrus neurons was completely preserved by TB treatment (fig. 18 A-C).

**TB treatment abrogates age-dependent memory and neuromuscular deficits in 3xTg mice**
The NORT discrimination index (DI) was significantly reduced in 17 month AD mice compared to 10 month AD mice (Fig 19 B). Likewise, the recognition index (RI) was also significantly decreased in 17 mo compared to 10-mo AD mice (Fig. 19 C). No significant differences between 2 and 10-mo AD mice were noted. However, the 10 mo AD mice performed slightly, but not significantly worse in short-term memory RI compared to 2 mo AD mice (Fig. 19 B). While 17-mo AD mice exhibited poor short-term memory in the NORT, TB treatment (17 mo AD+TB) increased their performance, exhibiting DI and RI levels similar to or better than in the 2-mo AD group. TB treatment led to improved preference for the novel object over the familiar object in 17-mo AD TB treated mice (Fig 19 A-C). In the Y-maze test, spatial memory and behavioral alteration showed an age-dependent alteration in spontaneous behavior, similar to NORT results. TB treatment significantly increased the performance of 16-mo AD mice compared to the untreated 16-mo AD group (Fig 19 D).

Consistent with the memory and behavioral studies, both muscular function and motor coordination declined with age in the 3xTg mice. Grip strength declined significantly with in AD mice with age compared to 2-mo B6.129 mice, with the 17-mo AD mice being most affected. Grip force was significantly lower only in the 17-mo AD mice. Both measures increased in the 17-mo AD group treated with TB (Fig 19 E,F). Performance on balance and coordination test using the RotaRod test varied widely in both the 2 and 10-mo AD mice, but the 17-mo AD mice exhibited decreased ability to maintain their balance and TB treatment increased the time to latency similar to that of the 10-mo AD group (Fig. 19 G).

Discussion

Current data determined that the dysbiosis associated with the AD progression in 3xTg mice was characterized by a loss of butyrate-producing bacteria (Fig. 11-13) and preceded the development of tau pathology (Fig. 15). This loss of butyrate-producing
bacteria leads to the lower concentrations of butyrate in the cecum, indicating that the
loss of the producers is resulting in a functional loss of butyrate for the host (Fig. 11 J).
Supplementing the loss of butyrate beginning at 6 months, a time after which Aβ
deposition has begun (Oddo, Caccamo, Shepherd, et al., 2003), prevented the dysbiosis
seen in the 3xTg mice at 18 months (Fig. 13-14). The distinction between the TB-treated
mice and controls was characterized by an increase of diversity, **Firmicutes**, and total
butyrate-producing families (Fig. 11 E). Specifically, **Lachnospiraceae** was significantly
increased, and this family includes many butyrate producers (Fig. 11 J) (Singhal et al.,
2021). These data show that by supplementing with butyrate, the GM was able to better
support the butyrate-producing bacteria and TB prevented the dysbiosis seen in the non-
supplemented animals. Restoring the loss of butyrate by the microbiome with TB did not
decrease the Aβ plaques at 18 months of age (Fig. 16 A, B). However, these mice,
despite having equivalent Aβ plaque burden, performed better in the cognitive
assessments including memory tests and muscular function tests (Fig. 19) indicating that
restoration of butyrate to the brain halted progression of AD in these 3xTg mice.
Clinically, a similar pattern has been described in which patients are deemed non-
demented with Alzheimer’s neuropathology, or NDAN (Arriagada, Marzloff, & Hyman,
1992; Bjorklund et al., 2012).

Current data may help to elucidate how NDAN subjects maintain cognitive
function in the face of neuropathogenic amyloidosis. A key step in AD-related protein-
induced neuroinflammation is the activation of microglia (Calvo-Rodriguez, Garcia-
Rodriguez, Villalobos, & Nunez, 2020; Dagher et al., 2015; Leng & Edison, 2021). Aβ
activates microglia through TLR4, initiating the inflammasome signaling cascade (Y. Liu,
Y. Dai, et al., 2020). In association with inflammation, microglial activation has also been
implicated in the development of oxidative stress through the APOE regulation of nitric
oxide (Colton et al., 2002). Oxidative stress is a critical component to pathogenesis and
predates the clinical diagnosis of AD in patients (Pratico et al., 2002). The environment created by oxidative stress and pro-inflammatory activation of microglia can enhance the phosphorylation of tau (Dias-Santagata, Fulga, Duttaroy, & Feany, 2007). Microglia aid in P-tau propagation through plaque-associated microglial releasing extracellular vesicles containing pathogenic P-tau from phagocytized neurons, which are taken up by other neurons to promote propagation of NFTs (Clayton et al., 2021; Stalder et al., 1999). The environment created by the continual activation of microglia plays a vital role in the development of NFTs. Post translational modifications of tau alter its folding (Andorfer et al., 2003; M. Liu et al., 2020). Phosphorylation plays a critical role in NFT development and hyperphosphorylated-tau is linked to NFT maturation and ultimately leads to cell death (Andorfer et al., 2003; M. Liu et al., 2020)). Hyperphosphorylated tau (D2Z4G and AT-8 co-staining, Fig. 15 C-F), which promote aggregation and the formation of paired helical filaments (Bibow et al., 2011; Jeganathan et al., 2008), was virtually prevented by TB treatment (Fig. 15 F). The reduction in tau hyperphosphorylation prevents the prion-like activity of the protein aggregates (Alonso, Beharry, Corbo, & Cohen, 2016; Puangmalai et al., 2020) and related cognitive decline. These data, taken together with the current study, indicates that targeting microglia activation may alleviate AD-related pathology.

The microbiome data indicated a functional loss of butyrate, which was targeted with TB. To prove that TB supplementation led to an increase in brain butyrate concentration, acetylation of H3K9/K14 was assessed in the hippocampus. H3K9/K14ac was significantly decreased in 12, and even further muted, in 18-month-old 3xTg mice (Fig. 18 A-C). The fact that TB supplementation strikingly increased hippocampal H3K9/K14ac in 18-month-old 3xTg mice, suggests that the concentration of butyrate increased to levels similar to the 2-month-old animals which was proven by one of its
functions as an HDAC inhibitor (Fig. 18 A-C). These data indicate that the decline in butyrate concentration in the CNS found in the 12 to 18-month-old 3xTg mice may play an important role in the pathologies associated with AD. The reduction of butyrate in the CNS coincided with the development of oxidative stress (Fig. 15 C-F), microglia activation (Fig. 15 A,B) and P-tau propagation (Fig. 16 C-F). Critically, butyrate concentrations arising from the GM were linked to the development of AD-like pathologies, as shown by the prevention of these pathologies in TB supplemented animals. Indeed, the increase in butyrate concentration did have an anti-inflammatory effect on microglia (Fig. 13 J) and has been shown to shift microglia to a neuroprotective state in traumatic brain injury (Huuskonen, Suuronen, Nuutinen, Kyrylenko, & Salminen, 2004; Patnala, Arumugam, Gupta, & Dheen, 2017). Butyrate has also been shown to limit the capacity of nuclear factor kappa B (NF-κB) to translocate into the nucleus, leading to less cytokine production (Inan et al., 2000). As a result of a reduction of cytokine production, there would be a decrease of oxidative stress (D. Yang et al., 2007). Butyrate also has been shown to upregulate the nuclear factor erythroid 2-related factor 2 (Nrf2) through dual acetylation of H3K9/K14 which alleviates oxidative stress (W. Guo et al., 2020; Ma, 2013). The current data indicates the loss of butyrate plays a critical role in the development of inflammation and oxidative stress, which provides the prime environment for the hyperphosphorylation of tau and targeting this loss is therapeutically appealing. In summary, the management of the GM and related product, butyrate, halted the progression of AD-like disease creating an NDAN-like population in the 3xTg model.

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Figure 11: Age-associated decrease in microbial diversity and distinct microbial composition observed in 3xTg mice through 16S analysis

Alpha diversity (A) OTUs and (B) Chao 1 Index measuring species richness and (C) Shannon Index; (D) Beta diversity PCoA plots generated using Weighted Bray-Curtis distances. Significant at 0.05 using a Benjamani-Hochberg correction for multiple
comparisons. Percentage relative abundance of Firmicutes and Bacteroidetes phyla (E) Firmicutes phylum, (F) Bacteroidetes phylum; and (G) F/B ratio. (H, I) butyrate-producing bacterial families belonging to Firmicutes phylum. Total butyrate-producing families in each age group (H) individual families shown in stacked bar graph (I) distribution of butyrate-producing families. Each color represents the same phylum in each group. (J) cecal butyrate levels. Data are represented as mean ± SEM (n = 4-6) with one-way ANOVA Tukey's corrected measures. Significance is denoted as *p < 0.05 and **p < 0.01. Student's t-test statistical analysis was performed and p <0.05 was denoted as ‘#’ when 2-month is compared to either 12-month or 18-month.
Figure 12: Alterations in the abundance of butyrate synthesizing genes in 3xTg mice

Heat map generated for 12 distinct butyrate synthesis genes identified by PICRUSt2 analysis for 2, 12, 18 and 18 month+TB groups. (A) The heat map shows genes from all four butyrate biosynthetic pathways, 4-aminobutyrate, acetyl-CoA, Glutarate, lysine, and common pathways, as well as the putative tributyrin esterase that hydrolyzes TB to produce butyrate. Red color signifies that indicated genes were absent or present in low abundance while green color signifies that those genes were present with intensity indicating the extent of abundance: puuE 4-aminobutyrate transaminase, 4Hbt- butyryl-CoA:4-hydroxybutyrate CoA transferase; phbB- acetoacetyl-CoA reductase, Thl or AtoB - thiolase, Ptb – phosphate butyryltransferase, Buk- butyrate kinase, Crt – crotonase, GctA -glutaconate CoA transferase (α), GctB – glutaconate CoA transferase (β), GctA – glutaconyl-CoA decarboxylase (α, β subunits), KamA – lysine-2,3-aminomutase, Bcd –
butyryl-CoA dehydrogenase (including electron transfer protein α, β subunits, ter1-trans-2-enoyl-CoA reductase (NAD+), and estA- putative tributyrin esterase.
Figure 13: Effects of TB on microbial diversity and distinct microbial composition observed in 3xTg mice.

Alpha diversity indices are shown – (A) Chao 1 Index measuring species richness and (B) Shannon Index measuring species abundance and evenness. (C) Beta diversity PCoA plots generated using Weighted Bray-Curtis distances are shown for 18-month,
and Tb-treated 18-month-old 3xTg mice. The Beta diversity data are significant at p<0.05 calculated using ADONIS. Percentage relative abundance of Firmicutes and Bacteroidetes phyla. The effect of oral administration of Tb are shown on (D) Firmicutes phylum; (E) Bacteroidetes phylum; and (F) F/B ratio. The change in percent relative abundance of total butyrate-producing bacterial families belonging to the Firmicutes phylum (G) and individual families (H) are shown in 18-month-old 3xTg mice with and without TB. In (H), each color represents the same phylum in each group. (I) Percent relative abundance of Lachnospiraceae family upon TB treatment is shown. (J) The increase in the cecal butyrate levels after oral administration of Tb were shown. Data in each graph represented as mean ± SEM (n = 4-6). The student t-test statistical analysis was performed, and significance denoted as *p < 0.05, and **p < 0.01.
Figure 14: Age-dependent phyla composition in 3xTg mice.

(A,B) Stacked bar graphs depicting the distribution of bacterial Phyla based on percent relative abundance in (A) 2, 12, and 18 month old 3xTg mice, or (B) 18 month old 3xTg mice either untreated (18 mo) or treated with TB (18 mo+TB). The taxonomic assignments of phyla were defined during 16S rRNA gene sequencing. Colors represent the same phyla in each group. (C) Beta diversity (depicting compositional differences) PCoA plot generated using Weighted Bray-Curtis distances for 2-, 18-, and 18-month treated with TB 3xTg mice. For each measure n=4-6
Figure 15: TB abrogates age-associated neuroinflammation and oxidative stress in 3xTg mice.

Representative fluorescent images of microglial activation in sagittal brain sections of the subiculum of 3xTg mice using IBA-1(FITC), co-stained for neurons (NeuN-Cy5) and
nuclei (DAPI) obtained using Keyence 810 microscope (Keyence, Itasca IL) at 20X magnification (A). The fractional area of IBA1-positive staining was quantified using Keyence 810 analyzer software (B). Representative images of IHC staining for 4-HNE visualized using DAB (3, 3'-diaminobenzidine). (C) or acrolein adducts visualized using DAB (3, 3'-diaminobenzidine). (E) Quantification of individual 4-HNE (D) or acrolein adduct (F) stained sections were counted in the striatum luciderm and molecular layer of the hippocampus (D,F). Magnification bar = 200 µm. (n=4-6) Bar graphs and error bars represented as means ± standard deviation, and data from individual mice are shown in closed circles. Statistical analysis is shown as Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
Figure 16: TB prevents hyper-phosphorylation of Tau in 3xTg mice.

Amyloid pathology was accessed in the subiculum by DAB (A) and florescent IHC (C). The presence of the Aβ40 or Aβ42 in the subiculum was assessed through IHC staining.
with D12B2 mAb visualized using DAB (3, 3′-diaminobenzidine). Representative images taken at 20X magnification (A) and absolute positive area quantification as in Figure 14 (B) are shown. To access tau hyper-phosphorylation, sagittal brain sections were triple stained with p-tau mAb (AT8-TRITC and D2Z4G-FITC) and NeuN-Cy5, and representative images are shown (C). The absolute positive area was determined for either AT8 (D), or D2Z4G (E) individually as well as for AT8 and D2Z4G co-localization (F). Magnification bar = 200 µm. (n=4-6) Bar graphs and error bars represented as means ± standard deviation, and data from individual mice are shown as closed markers. statistical analysis is shown as Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
Figure 17: TB reduces co-expression of hyperphosphorylated tau within neurons of the subiculum of 3xTg mice and astrocyte activation in the subiculum not significantly reduced by TB supplementation

(A) Representative confocal microscopy images of fluorescent IHC staining for markers of phosphorylated tau, AT8 (red) and D2Z4G (green), were significantly increased within subiculum NeuN-positive (purple) neurons of 3xTg mice by 18 months. This co-localization was reduced in 18mo+TB. Lower horizontal bar represents the xz-axis; right vertical bar represents the xy-axis. White box is magnified in (B). Size bar = 100 µm.
GFAP-FITC assessment by florescent IHC (C) and quantification of GFAP (D). Size bar = 200 µm. (n=4-6) Bar graphs and error bars represented as mean scores ± standard deviation, and data from individual mice are shown in open circles. Statistical analysis is shown as Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
Figure 18: TB supplementation prevents the age-related decrease of H3K9/K14ac in 3xTg mice.

Representative images of K9/K14 acetylation of histone 3 in the hippocampus of indicated 3xTg mice were assessed by florescent IHC using anti-H3K9/K14ac-FITC and NeuN-Cy5 (A). Quantification of total H3K9/K14ac staining indicated as absolute positive area staining (n=4-6) (B). The percent of neurons positive for H3K9/K14ac staining in the dentate gyrus was determined by H3K9/K14ac-FITC and NeuN-Cy5 co-localization/total NeuN-Cy5 staining (C). scale bar = 200 µm. (n=4-6) Bar graphs and error bars.
represented as means ± standard deviation and data from individual mice are shown in open circles. Statistical analysis is shown as Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
Figure 19: TB protects 3xTg mice from developing memory and neuromuscular deficits as 17-month-old mice

Short-term episodic and spatial memory were measured in 17-mo 3xTg mice using NORT (A,B) and Y-maze test (C,D), respectively. (A) Cartoons depict representative travel traces during the NORT probe stage from indicated groups of 3xTg mice. The yellow circle indicates placement of the novel object and its surrounding area, and the red lines trace the route of travel individual mice took during the 5-minute recording time. (B) Calculated NORT recognition indexes (RI) indicating a lost preference in the 17-mo 3xTg mice for the novel object arena, and 17-mo+TB treatment retained novel object recognition. The spontaneous alternation in the 3-arm Y-maze trial (C) is shown as percent spontaneous alternation and calculated as the number of alternations between
the 3 open arms of the apparatus divided by the total number of arm entries multiplied by 100. (D) The percent alternation between arms in the 2-arm Y-maze trial.

Neuromuscular function measurements were determined by measuring the force with which the front limbs no longer clasp a wire mesh and depicted as (E) raw grip force (N), or as (F) grip strength, calculated as grip force/total body weight (g). The time to fall (latency) was measured in seconds for mice placed on an accelerating rotarod apparatus (G). Bar graphs and error bars represent means ± standard deviation (n=5-6), and data from individual mice are shown in closed circles. Statistics calculated using one-way ANOVA with Tukey post-hoc comparisons test with single variance and are indicated as: ns—not significant, *p <0.05, **p<0.01, ***p<0.001, ****p < 0.0001.
CHAPTER 4:

ROLE OF ALDOSE REDUCTASE IN HEPATIC AND BRAIN INFLAMMATION ALONG THE LIVER-BRAIN AXIS

Introduction

Host health and its symbiotic relationship with the GM are intimately connected. If the gut microbiome loses beneficial bacteria, increases pathogenic species, and/or loses diversity, that dysbiosis can lead to pathological effects for the host (DeGruttola, Low, Mizoguchi, & Mizoguchi, 2016). One of the hallmarks of pathogenic dysbiosis are an increased in gram-negative bacteria, gut-barrier dysfunction, and translocation of inflammatory bacterial products across the gut barrier into the host circulation (Genua, Raghunathan, Jenab, Gallagher, & Hughes, 2021). Lipopolysaccharide (LPS), a gram-negative bacterial product, induces production of pro-inflammatory cytokines through toll-like-receptor (TLR) 4 activation (Alpizar et al., 2017; X. Chang et al., 2021). These cytokines can induce the production of reactive oxygen species (ROS) and this process occurs in both immune cells and endothelial cells, resulting in oxidative stress and a negative-feedback loop inducing more cytokine production (Padgett, Broniowska, Hansen, Corbett, & Tse, 2013; Simon & Fernandez, 2009). As a result of increased oxidative stress, lipid peroxidation can occur, leading to increased lipid aldehydes which can damage DNA, proteins and eventually lead to cell death (Su et al., 2019).

These GM-derived events will induce CNS neuroinflammation and have been implicated in several neurodegenerative diseases and/or neuropsychiatric symptoms such as depression, anxiety, and fatigue (Batista, Gomes, Candelario-Jalil, Fiebich, & de
Oliveira, 2019; Neuendorf, Harding, Stello, Hanes, & Wahbeh, 2016). Sustained LPS exposure through a leaky gut results in endotoxin tolerance in the peripheral circulation, but in the CNS leads to prolonged expression of pro-inflammatory cytokines (Thomson, McColl, Cavanagh, & Graham, 2014). As a result of these inflammatory events, microglia (the resident CNS macrophages) will recruit peripheral leukocytes - natural killer cells and neutrophils - that infiltrate the CNS (H. He et al., 2016). The infiltrating neutrophils in particular are thought to play a role in the neuropsychiatric symptoms previously mentioned (H. He et al., 2016). The critical immune response is initially beneficial, but when sustained can become deleterious (Gupta, Kunnumakkara, Aggarwal, & Aggarwal, 2018). To better understand the underlying mechanism(s) involved, the inflammatory events associated with dysbiosis can be mimicked or exaggerated by a high dose of LPS injected intraperitoneally (IP) into mice.

Aldose reductase (AR) is an enzyme in the glucose polyol pathway that metabolizes glucose to fructose (Hyndman, Bauman, Heredia, & Penning, 2003). The polyol pathway utilizes glucose in activated macrophages presented with an increase of sorbitol, which is an intermediate product in fructose production mediated by AR (K. C. Chang et al., 2013). The increase in fructose production by AR requires nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor (Hyndman et al., 2003). The consumption of NADPH will then limit the capacity of the cell to process lipid aldehydes, increasing oxidative stress (Srivastava, Ramana, & Bhatnagar, 2005). Another function of AR is the reduction of glutathione-bound 4-hydroxynonenal (GS-HNE) to glutathionyl-1,4-dihydroxynonene (GS-DHN), which has been implicated as a driver of cytokine expression (Ramana, Bhatnagar, et al., 2006). These functions of AR make it a critical mediator of oxidative stress and inflammation. Indeed, its inhibition reduces the inflammation in response to LPS by downregulating nuclear factor kappa B (NF-κB).
translocation and related cytokine production (Ramana, Reddy, Tammali, & Srivastava, 2007; Ramana & Srivastava, 2006). AR inhibition has been proposed as a therapeutic intervention for non-alcoholic fatty liver disease, alcohol-associated liver disease, and diabetic complications (Kovacikova et al., 2021; Qiu & Chen, 2015; M. Wang et al., 2020).

Here, we identify AR as a critical protein in endotoxin-driven CNS neuroinflammation and describe its contribution to these gut-driven inflammatory events that may be related to the development of neurodegenerative diseases and neuropsychiatric symptoms.

Methods

Animal model:

8-12 weeks C57BL/6 mice or AR knockout (Akr1b3−/−, ARKO) (n=5-9) were subjected to stimulation for 16 hours with or without LPS (3.0 mg/kg body weight) through intraperitoneal injection.

Tissue collection:

Mice were anesthetized with avertin (2,2,2-tribromoethanol in 0.02 ml of 1.25% 2-methyl-2-butanol in saline, Sigma Aldrich), and blood was drawn from the inferior vena cava. Mice were then transcardially perfused with 20 ml PBS and tissues were harvested.

Immunohistochemistry:

Mice were transcardially perfused with 20 ml PBS, followed by 15 ml of 4% paraformaldehyde (PFA) in PBS, pH 7.4. Brains were dissected and additionally fixed overnight at 4°C in 4% PFA. Then, brains were transferred to 30% sucrose solution and
stored for 3 days at 4°C. Following sucrose dehydration, half of the brain was used for paraffin embedding. After embedding, the tissue was sectioned 10 um thick. After sectioning, mounted slides were incubated at 37°C for 48 hours. For paraffin removal, hydration, and antigen retrieval, slides were immersed in trilogy (Millipore Sigma, St. Louis, MO) and incubated in a pressure cooker at high pressure for 12 minutes. Both paraffin and cryo-embedded tissues were blocked in TBS + 0.1% Triton X-100, 0.5% BSA, and 10% normal donkey serum for 1 hour at room temperature and then incubated overnight at 4°C with primary antibodies in blocking buffer, followed by incubation in secondary antibodies at room temperature for 1 hour. The slides were imaged on a Keyence BZ-X810 (Keyence Corporation of America, Itasca, IL). Fluorescence intensities were measured with BZ-X810 analysis software (Keyence Corporation of America, Itasca, IL). Serial sections (n=3-5) of were used to determine an average value for each animal. Values were only removed for damaged or folded tissues that prohibited accurate analysis. For each antibody, an isotype and no primary were used as controls. See data table 2 for antibody information.

Mononuclear cell isolation:

Brains were harvested from mice transcardially perfused with 20 ml PBS, washed in 1 mL of PBS, minced in Liberase LT in HBSS with Ca²⁺ and Mg²⁺ (Millipore Sigma) and incubated at 37°C for 30 minutes. Tissue was then forced through a 70 μm filter and washed with 3 mL PBS+10% FBS with DNase followed by 10 mL of PBS+10% FBS. Myelin was removed using 33% Percoll plus (Millipore Sigma) and washed with 10 mL of PBS+10% FBS.

Flow Cytometry:

Brain cell suspensions were incubated for 5-10 minutes with 1 μg/sample anti-CD16/32 mAb (clone 2.4G2, BioLegend, San Diego, CA) to block non-specific Ab binding to FcR (FcyR III/II) and then stained with fluorochrome-labeled antibody panels.
Cells were then washed in FACS staining buffer (SB: 1X PBS containing 2% FBS and 0.02% sodium azide) then 1X PBS and fixed in 2% methanol-free formaldehyde (Polysciences, Warrington, PA) for 2-24 hours before acquisition on a BD FACSCanto II flow cytometer, (BD Biosciences, CA)

The following monoclonal antibodies were used: CD11b (Clone: M1/70), FITC, eBioscience; CD45 (30-F11), PerCP-Cyanine5.5, eBioscience; Ly-6G (1A8-Ly6g), PE, eBioscience; Ly-6C (HK1.4), PE-Cyanine7, eBioscience). Panel-specific fluorescence minus one (FMO) controls on spleen cell populations were used to define negative events in gating strategies (16888771 (Maecker & Trotter, 2006)). In select experiments, enumeration of liver cell preparations was performed using comparison to Precision Count beads (BioLegend) per the manufacturer’s protocol.

**Cytokine analysis:**

Brain and liver tissues were homogenized in a buffer containing 20 mM Tris, 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, 1% Triton X-100, and a cocktail of protease inhibitors (Sigma) (Fox et al., 2005). The levels of various cytokines/chemokines were measured using MSD (Meso Scale Diagnostics, Rockville, MD) kits.

**Tissue culture:** The RAW264.7 macrophage cell lines were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were treated with LPS (10 and 100 ng/ml) and Epalrastat (an AR inhibitor, 50uM) pretreatment for 30 min.

**Statistical analysis:**

Statistical analysis was performed using GraphPad Prism version 9 for Windows (GraphPad Software, Inc., La Jolla, CA). Data were analyzed by unpaired analysis of variance (ANOVA) with Bonferroni posttest analysis (for greater than two groups). Differences were considered statistically significant at p<0.05.
Results

LPS drives PMN infiltration into both Liver and Brain which is attenuated by ARKO.

I.P. LPS injection incited a robust immune response. The genetic removal of AR significantly blunted this response. The administration of LPS led to a robust infiltration of PMNs into the liver assessed by CAE staining and FACS (40.4 neutrophil/1000 hepatocytes) (27% of CD45+ cells) (Fig. 19 A). ARKO mice had significantly less LPS-induced PMN infiltration into the liver which reduced the neutrophils to 21.1 per 1000 hepatocytes (CAE) and 12% of immune cells (FACS) (Fig. 20 B). Along with the changes in PMNs, there was a significant change in some of the cytokines and chemokines. Levels of GM-CSF, IP-10, IL-17a, IL-6 and TNF-α were all reduced in concentration in ARKO+LPS mice when compared to WT+LPS (Fig. 21). Thus, hepatic inflammation is significantly impacted by deletion of AR. Coinciding with increased hepatic inflammation, there was an increase in many circulating cytokines. In the WT+LPS mice, the following cytokines were all increased: GM-CSF (14.75 pg/ml), IFN-γ (420.1 pg/ml), IL-10 (910.8 pg/ml), IL-1β (31.5 pg/ml), IP-10 (3,607.2 pg/ml), IL-17a (44 pg/ml), IL-6 (36,548.5 pg/ml) and TNF-α (149.4 pg/ml) (Fig. 21). In the ARKO+LPS, GM-CSF (1.5 pg/ml), IL-10 (551.2 pg/ml), IL-17a (5.4 pg/ml), IL-6 (4,518.4 pg/ml), IP-10 (2215 pg/ml) and TNF-α (45.7 pg/ml) were reduced when compared to the WT+LPS (Fig. 22 A,C,D,G, H). There was also a lower average concentration of IL-1β (7.9 pg/ml) and IFN-γ (27.6 pg/ml) but they did not reach statistical significance. Considering the increase in circulating cytokines and hepatic inflammation, the inflammatory status of the brain was evaluated. As with the liver, we also noted an increase in PMN infiltration into the brains of WT from 5% PMNs to mice treated with LPS to 10% PMNs of 45CD+ (Fig. 20 D,E). The ARKO+LPS mice had a considerable abrogation in the percentage of PMNs in the brains of ARKO+LPS mice (5.9% PMNs of CD45+), remaining at levels
similar to the non-treated mice (Fig. 20 D,E). These data indicate that the genetic ablation of AR abrogates the systemically driven neutrophil infiltration into the brain. ARKO mice exhibit differential production of cytokine but similar microglial activation in response to LPS.

Immunohistochemical analysis indicates that microglia became activated in response to i.p. LPS injection (Fig. 24 A,B). Once activated, Microglia secrete IL-1β and TNF-α in response to LPS (Y. He, Taylor, Yao, & Bhattacharya, 2021). The LPS-induced activation was evident in both WT and ARKO animals elevating from the untreated averaging from 8,324.9 μm to 24,196.2 μm in the LPS groups (Fig. 24 B). IP administration of LPS induces cytokine concentration in the brain to increase secondary to inflammasome activation (Dolunay et al., 2017). Data from the brains of WT mice treated with LPS demonstrated a significant increase in IL-1β (0.5 pg/g) and TNF-α (0.2 pg/g) (Fig. 22 C,H). Notably, ARKO mice treated with same amount of LPS had significantly reduced concentrations of IL-1β (0.1 pg/g) and TNF-α (0.1 pg/g) as compared to their WT counterparts (Fig. 23 C,H). Other cytokines measured, GM-CSF, IFN-γ, IL-10 and IL-17a were not significantly different in the ARKO+LPS mice (Fig. 23 A,B,E,F). Interestingly, IP-10, an IFN-γ driven cytokine, was significantly reduced from 62.3 pg/g in WT+LPS to 31.6 pg/g in ARKO+LPS (Fig. 23 B,G).

Impact of AR inhibition on macrophage LPS-induced cytokine production

In RAW cells, pretreatment with EPAL blunted the response to LPS as seen by a significant reduction in cytokine gene expression. TNF-α, IL-6 and IL-1β were all significantly reduced with inhibition of AR (Fig. 25 A,B,C). The resolving cytokine and immunosuppressive cytokine IL-10 did not increase (Fig. 25 D).
Discussion

Current data identified AR as a critical mediator of LPS-induced inflammation. Other studies in endothelial cells have shown that AR inhibition lowers the expression of the adhesion molecules, ICAM-1 and VCAM-1 (Ramana, Bhatnagar, & Srivastava, 2004). These proteins are critical for endothelial cell recruitment into the parenchyma of organs (Banks & Robinson, 2010). The genetic ablation of AR likely prevented adhesion proteins from being expressed, therefore preventing neutrophil infiltration not only into the liver but also into the CNS (Fig. 19 A-E). Along with the prevention of neutrophil infiltration, significant increases in cytokine concentrations were blocked, and these effects were likely controlled by AR (Fig. 20). Ethanol consumption leads to the increase in AR expression in the liver (M. Wang et al., 2020) and a hallmark of alcoholic liver disease is the infiltration of neutrophils into the liver. Collectively, the data suggests that AR inhibitors may be therapeutically useful to prevent the sustained infiltration of neutrophils into the liver that results from ethanol abuse.

The expression of ICAM and VCAM is not only critical in the liver but also in the blood brain barrier (BBB). The endothelial cells of the BBB are also capable of upregulating these adhesion proteins in response to activation (Zameer & Hoffman, 2003). The circulating LPS injected in our study could directly, or indirectly through upregulated cytokines, lead to activation of BBB endothelial cells (McHale, Harari, Marshall, & Haskard, 1999). The upregulation would increase the recruitment of neutrophils, which was evident in WT+LPS mice but was notably absent in the ARKO+LPS cohort (Fig. 19 A, B). Along with the increase in adhesion molecules on endothelial cells, IL-17a is known to affect the integrity of the BBB (Ni et al., 2018). The significant increase in plasma of IL-17a in the WT+LPS mice which would have a deleterious effect on the BBB, making it more permeable to neutrophils. These data
indicate that gut-derived inflammation secondary to dysbiosis, along with the subsequent release of bacterial products, which if sustained could potentiate the extent of neuropsychologic diseases (Simpson et al., 2021) may be alleviated through the inhibition of AR. An AR inhibitor may protect the BBB by reducing IL-17a as well as reducing endothelial cell expression of adhesion proteins and their activation. Thus, the recruitment of neutrophils could be prevented and could potentially alleviate neuropsychological symptoms associated with the infiltration and inflammation.

Interestingly, while the neutrophil infiltration in the brain was prevented in the ARKO+LPS mice, microglial activation was sustained (Fig. 19 C-E). When comparing the inflammatory cytokine in the brains of WT+LPS and ARKO+LPS there are some significant differences, but not all the cytokines are reduced (Fig. 22). In the ARKO+LPS brains, the typical microglial/macrophage cytokines in response to LPS, IL-1β and TNF-α, were significantly lower (Fig. 22 C,H). Thus, deletion of AR allowed for an increase in IBA-1 but prevented the expression of these proinflammatory cytokines. Similarly, Raw 264.7 cells treated with EPAL showed significant reduction in the TLR4/NF-κB related cytokines (Fig. 24 A,B). These data replicate findings that NF-κB-related proteins are down regulated when AR is inhibited (Ramana & Srivastava, 2006). Of note, increased IFN-γ concentrations were found in the brains of both WT+LPS and ARKO+LPS cohorts, as well as the downstream protein IP-10 (Fig. 22 G). The expression of IP-10 indicates that while ARKO mice have a limited capacity to produce NF-κB-related proteins, the STAT1 pathway remains unaffected. The microglia activation seen by IBA-1 upregulation likely is a response to IFN-γ, inducing the microglia to produce IP-10 (Fig. 22 G). These data indicate that the immune activation in response to LPS is significantly impacted and the use of an AR inhibitor could abrogate/attenuate NF-κB related inflammation.
Dysbiosis continues to be implicated as a component in the pathogenesis of many of chronic neurological diseases and to be related to inflammation driven by translocated bacterial products (Simpson et al., 2021; Sochocka et al., 2019; J. Wang, Chen, & Wang, 2020). Current data suggest that this inflammation could be therapeutically targeted by AR inhibitors to alleviate some of the symptoms related to the inflammation driven by the microbial products such as LPS. EPAL was effective in clinical trials of diabetic peripheral neuropathy (Hotta et al., 2006) and is well tolerated with minimal or manageable side effects. We suggest that targeting AR inhibition in CNS diseases characterized by LPS-induced inflammation may be an effective therapeutic strategy.

Acknowledgements
A thanks to: Min Wang for aiding in animal care and treatment and performing the Raw 264.7 experiment; Jing Zhang for staining CAE.
Figure 20: Genetic deletion of AR reduces the LPS-driven neutrophil infiltration to the liver and brain.

WT and ARKO mice were LPS injected, and tissues were collected 16 hours later. Images were obtained using Keyence bz-18 microscope at 20X magnification A) CAE staining of neutrophils indicated by the red staining in the liver. B) quantification of A:
neutrophils hand counted per 1000 hepatocytes by a double blinded volunteer. C) Gating strategy for identification of neutrophils used in D and F. D) Brain mononuclear cells gated for neutrophils E) quantification of D. (n=4-6) statistical analysis is shown as Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
Figure 21: Genetic deletion of AR reduces LPS driven cytokine concentrations in the liver.

WT and ARKO mice were LPS injected, and tissues were collected 16 hours later. After PBS perfusion and tissue collection, hepatic lysates were made and analyzed using MSD multiplex assays. A-H) multiplex MSD assay results, normalized to weight of hepatic tissue. (n=4-6) Statistics were calculated using non-parametric one-way ANOVA with multiple comparisons, and significance indicated as *p<0.05, **p<0.01, ***p<0.001, ****p < 0.0001.
Figure 22: Genetic deletion of AR reduces the majority of LPS-driven cytokines circulating in the plasma.

WT and ARKO mice were LPS injected, and tissues were collected 16 hours later. Before PBS perfusion blood was drawn with heparin and plasma isolated and then analyzed using MSD multiplex assays. A-H) multiplex MSD assay results. (n=4-6) statistical analysis is shown as Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
Figure 23: Reduction in cytokine expression in the brain of ARKO mice treated with LPS.

WT and ARKO mice were LPS injected, and tissues were collected 16 hours later. After PBS perfusion and tissue collection, brain lysates were made and analyzed using MSD multiplex assays. A-H) multiplex MSD assay results, normalized to weight of brain tissue. (n=4-6) statistical analysis is shown as Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
Figure 24: Microglia activation is present in the hippocampus of all mice treated with LPS.

WT and ARKO mice were LPS injected, and tissues were collected 16 hours later. After PBS perfusion, 4% formaldehyde was perfused, tissue harvested and post fixed overnight in 4% formaldehyde and processed for paraffin embedding. Images were obtained using Keyence bz-18 microscope at 10X magnification. A) Brains FFPE were
stained with IBA-1(FITC) after antigen retrieval. B) Magnification of A location indicated from the white boxes in A. C) Area IBA-positive staining was quantified using BZ-810 analyzer software (n=4-7) statistical analysis is shown as Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
Figure 25: Raw 264.7 cells treated with AR inhibitor, EPAL, have blunted reaction to LPS stimulation.

Pretreatment for 30 min with or without EPAL (50 µmol), then stimulated with LPS (10 or 100 ng) for 24 hours. From these cells isolation of mRNA and process for qRT-PCR. A) TNF-α expression pattern. B) IL-1β expression pattern. C) IL-6 expression pattern. D) IL-10 expression pattern. (n=3) statistical analysis is shown as Kruskal Wallis analysis p value significance: p value < 0.05 = *, p value < 0.01 = **, p value < 0.001 = *** and p value < 0.0001 = ****.
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CHAPTER 5:

FINAL DISCUSSION

Chapter 2: NOVEL FMT for the study of ethanol-induced dysbiosis

As our ability to analyze the gut microbiome (GM) and its metabolites increases, we are starting to grasp its significant impact on health and disease. A novel approach to fecal microbial transplantation (FMT) was developed to study the GM's role in the pathogenesis of alcoholic associated hepatitis (AH) and delineate the related neuropathology. Moreover, the model is widely applicable and may be adaptable to fit other diseases or organ injury models. Importantly, the capacity to transplant the GM into conventional mice avoids the pitfalls of germ free (GF) animals (as introduced in chapter 1). The adaption of the FMT model to other disease paradigms such as the 3xTg animal model for Alzheimer’s disease (AD) may help the model to better phenocopy the disease (such as AD, see also chapter 3).

GM dysbiosis, functional loss of butyrate, and related pathogenesis

The FMT model allowed us to determine that the GM plays a role in the development of neuropathology in mice with or without the consumption of ethanol. The GM modulated by ethanol was characterized and target the specific functional losses. Through metagenomic analysis the dysbiosis was characterized by the functional loss of butyrate-producing bacteria families, Lachnospiraceae and Ruminococcaceae. The dysbiosis also led to decrease in cecal butyrate concentration in the FMT animals. This loss of butyrate in the cecum not only confirms that the FMT was successful, but also
shows that there is functional loss of short chain fatty acids (SCFA). That SCFA loss has significant impact on the health of the gut barrier due to its function in maintaining the mucus layer and promoting better tight junction protein expression (Peng et al., 2009). In combination with the immediate effect of ethanol on the gut barrier (Elamin et al., 2014), the loss of butyrate has a strong negative effect on barrier integrity. The bacterial products that escape the GM across the intestinal barrier subsequently modulate the peripheral immune system and lead to liver injury. From our AH-FMT, the immune cell was modulated by the T-cell polarization shifts towards TH17 cells, coinciding with a significantly increase in IL-17a protein expression. These events in the periphery can then affect the CNS.

Pathogenic inflammation induced by dysbiosis and ethanol in the CNS, particularly the cerebellum

Various ethanol feeding models including the chronic plus binge and binge models, are well documented to produce increases in astrocyte and microglia activation (Fonseca, Alves, Carrondo, & Santos, 2001; Lowe et al., 2018). However, these studies focused on the cortex or hippocampus. In the sub-acute model, astrocyte activation showed regional differences (Fig. 5 C-F). Of particular interest was the cerebellum, which unlike the prefrontal cortex and hippocampus, was specifically sensitive to modulation of the GM with AH-FMT (Fig. 8 A-D). From this experiment, the regional differences in inflammation led to differences in neuronal ERSR. The dysbiosis induced by ethanol affects the Purkinje cells (PCs) in the cerebellum causing them to increase expression of ERSR-related proteins. Ethanol also enhanced cerebellar chaperon protein expression, but the AH microbiome was necessary to increase expression of XBP1 and CHOP in the prefrontal cortex and hippocampus. Critically, cerebellar PCs showed differential expression of ERSR proteins, notably not expressing XBP1 in the AF+AH-FMT mice (Fig. 8). MRI studies of patients with AUD show that the PCs are
specifically affected in the cerebellum. Initially it was believed that there was a loss in the number of PC, but recent data suggest rather that the PCs shrink, losing volume (Andersen, 2004). Damage to these cells leads to ataxia, which is common for patients suffering from withdrawal (Trevisan et al., 1998). Our data provide a potential mechanism as to how the PCs are affected in these patients.

Effects of AH-FMT-related inflammation on microglia, cytokine concentration, and immune system infiltration

The lack of microglia activation was surprising in this study. In every treatment from our FMT experiments, there were no indications of microglial activation. Given the presence of enhanced expression of markers indicating neuronal dysfunction and astrocyte activation, microglia activation was expected but was not detected. In one of our recent papers, mice on 4 weeks of ethanol feeding did show microglial activation, indicating our FMT experiment is in the early stages of inflammation which is also indicated from the PMN infiltration (Fig. 3 E-G) (Avila et al., 2017). In traumatic brain injury, the initial immune response is an increase of multiple cytokines that drive neutrophils into the brain as the first responders (McKee & Lukens, 2016). To further characterize FMT-driven inflammation, a multiplex meso-scale discovery enzyme-linked immunoassay for the following proteins: TNF-α, GM-CSF, INF-γ, IL-10, IL-17a, IL-1β, IL-6, IP-10, KC/GRO, and MCP-1, showed that only IL-17a to be significantly changed (Fig. 3 H). These data indicate that this inflammatory response is still in the initiation phase and increasing the extent in ethanol consumption would increase expression in other cytokines beyond IL-17a. Consistent with this hypothesis, previous experiments and others have seen increased cytokine expression with extended ethanol feeding (Avila et al., 2017; J. He & Crews, 2008; Lowe et al., 2018). Nonetheless, IL-17a is an important cytokine that increases neutrophil chemotaxis (Kono et al., 2011) and our
FACS data indicate that the PMNs did infiltrate into the CNS, an effect driven by the increase in IL-17a in the CNS. The analysis of the inflammation in our model indicates early stages or low-grade inflammation. The experiment provides insight to the initiation of dysfunction; if consumption were allowed to continue it would lead to a progressive increase in inflammation and further glial activation and neuronal dysfunction.

Pathogenic changes related to AH-FMT in the prefrontal cortex

Our data from the prefrontal cortex and hippocampus may connect to other pathologies seen in patients with AUD. Understanding the mechanisms behind addiction has become an important aspect when treating patients with AUD. The prefrontal cortex has become an area of interest in understanding addiction (Goldstein & Volkow, 2011). The ERSR in the neurons induced by the AH-FMT with ethanol consumption may contribute to the alterations in neurochemistry that enhance drug-seeking behavior and addition.

Pathogenic changes related to AH-FMT in the hippocampus

Ethanol abuse is related to the development of dementia (Rehm, Hasan, Black, Shield, & Schwarzinger, 2019), as those who drink to the point of loss of consciousness are at a higher risk of developing dementia (Kivimaki et al., 2020). Our data in the hippocampus suggest that a dysbiotic microbiome may play an important role in the initiation of neuronal dysfunction (Fig. 7). In particular, the development of ER stress in the dentate gyrus, which is the site for neurogenesis and aids in memory formation, may relate to the cognitive decline (Abbott & Nigussie, 2020). Interestingly, data from chapter 3 show that oral butyrate supplementation elicited evidence of HDAC inhibition in the brains of the 3xtg mice. These data indicate that oral tributyrin administration led to increased concentration of butyrate in the CNS, agreeing with published data indicating
that butyrate can cross the blood brain barrier (Stilling et al., 2016). One of the inflammatory events that occurred in our model was the activation of astrocytes, both in the hippocampus and cerebellum. AF-AH-FMT, recipients of oral tributyrin had reduced astrocyte activation in the cerebellum (Fig 9 O,R). Butyrate is beneficial for astrocytes, as it regulates the bioenergetics of astrocytes and affects transcripts related to neuroinflammation (T. Yang et al., 2018). Butyrate also improves the viability and mitochondrial function in primary astrocytes (C. Wang, Zheng, Weng, Jin, & He, 2022). Through butyrate supplementation, astrocytes reduce expression of cytokines and TLR4 (T. Yang et al., 2018). The data imply that microbiome-produced butyrate plays a role in the activation state and inflammatory status of astrocytes. The restoration of butyrate seems to have a protective effect against alcohol, likely through the regulation of cytokines. Specifically, the identification of IL-17a as a critical mediator of the pathogenesis was significantly reduced in the brains of mice supplemented with tributyrin.

Effect of tributyrin supplementation on AF-AH-FMT-related ERSR and future studies

Similarly, when AF-AH-FMT were treated with TB, the expression of ERSR-related proteins were prevented in the brains of these animals in both the hippocampus and cerebellum (Fig. 9 H-T). These proteins were trending downward in the prefrontal cortex, but levels were not significantly lower. Interestingly, oral administration of butyrate reduces CHOP expression, which, under conditions of sustained expression, can lead to apoptosis (Kushwaha et al., 2022; Y. Zhao et al., 2021). Another study indicated that FMT with a healthy microbiome or treatment with butyrate resulted in a reduction of neuropathic pain (Bonomo et al., 2020). These studies and the data from chapters 2 and 3 indicate that butyrate can beneficially impact neuronal function and ameliorate ER stress. Importantly, while there was a significant increase in the ERSR in
neurons, these changes did not induce neuronal death. The lack of death connects to patient MRI data which suggest that the changes in volume can be reversed, but without improving cognitive impairment (Bartels et al., 2007; Gazdzinski, Durazzo, & Meyerhoff, 2005). Thus, the functional changes as a result from ERSR may permanently affect the neurons without leading to apoptosis.

Future studies will determine if this hypothesis is correct by assessing the role of AH-FMT on addiction and cognitive impairments, with behavioral studies connecting these behavioral changes to ERSR related changes in the neurons of the prefrontal cortex and hippocampus.

Hepatic encephalopathy is another mechanism by which the GM effects the CNS

In severe liver damage, patients can develop hepatic encephalopathy (HE) (Swaminathan, Ellul, & Cross, 2018). HE affects roughly 40% of cirrhotic patients who present with neurological symptoms, and it affects both cognition and neuromuscular function contributing to the repeated rehospitalizations and poor prognosis (Swaminathan et al., 2018). Interestingly, one of the patients AH whose stool was used for the FMT did present with HE. The pathogenesis of HE is that the compromised liver is unable to metabolize nitrogenous waste products derived from the GM (Swaminathan et al., 2018). Our GM data from those animals transplanted with the AH-FMT support the idea that tributyrin can not only provide butyrate but also act as a beneficial modulator of the microbiome (Fig. 2). The data from Chapter 3 on the 3xTg animals showing improvement of the neuromuscular function/strength and cognitive function, suggest that tributyrin supplementation would also likely be of benefit in treatment of these patients.
Conclusion from chapter 2

The experiment provides data that suggest that the management of the GM and tributyrin supplementation may be an important addition to the standard of care for patients with AUD and may provide therapeutic efficacy for those who develop HE.

Chapter 3: dysbiosis in the 3xTg model of AD

Aging inherently leads to an undefined dysbiosis and preventing or addressing this dysbiosis has become an area of substantive research (Ragonnaud & Biragyn, 2021). However, patients with diseases such as AD develop a more specific dysbiosis that are specifically characterized by a loss of butyrate-producing bacteria (Haran et al., 2019). Therefore, to determine if the 3xTg model produced the same loss of butyrogenic potential seen in AD patients, these mice were allowed to age to 18 months. Confirming our hypothesis, these 3xTg mice in our facility did develop dysbiosis that was characterized by a decrease in diversity and the loss of butyrate-producing bacteria at 12 and 18 months (Fig. 10 E-I). Specifically, these mice had a significant loss of Firmicutes, which significantly impacted the F/B ratio and resulted in a decrease in the family Ruminococcaceae (Fig. 10 G). Ruminococcaceae is one of the major butyrate-producing families (Vital, Karch, & Pieper, 2017). Along with the decreases in butyrate producers the cecal concentration of butyrate in the 12- and 18-month animals was lower (Fig. 10J). These data suggest that the characterization of dysbiosis in 3xTg is similar to that seen in AD patients. In particularly at 18 months, these animals show similar neuropathology and behavioral deficits (Fig. 18) to AD patients (Sterniczuk et al., 2010). The changes in the microbiome indicate that the 3xTg animals lose butyrogenic potential at 12 months, the time at which neurofibrillary tangles form (Oddo, Caccamo, Shepherd, et al., 2003) and preceding the major behavioral deficits (Fig. 10 H). This
indicates that the prolonged loss of butyrate is in part pathogenic and aids in the development of the cognitive decline.

**AD specific pathological markers: Aβ plaques and P-tau**

The development of the pathological Aβ plaques and hyperphosphorylation of tau (P-Tau) in the 3xTg were significantly increased at 18 months of age (Fig. 15 C-F). Data not only showed phosphorylation of tau but hyperphosphorylation, as the colocalization of the 2 antibodies used can recognize up to 5 sites on the tau protein. Oral administration of TB did not significantly reduce the individual markers of P-Tau, but the co-staining, indicative of hyperphosphorylation, was significantly reduced (Fig. 15 F). The interesting thing was that the increase of Aβ likely preceded the drop in butyrate producers. These data, along with the activation of microglia, suggest that the Aβ plaques initiate an immunological response that creates an environment supportive of the phosphorylation of tau. The functional loss of butyrate is directly associated with a pathogenic environment. To prove this point, intervention at 6 months after Aβ plaques had started forming and the environment had begun to shift towards a pathogenic one provided evidence that loss of butyrate is critical for the pathogenesis (Oddo, Caccamo, Shepherd, et al., 2003). The oxidative stress and inflammation progress was halted by butyrate supplementation, and while this changed the environment, it did not promote the phosphorylation of the tau.

**Pitfall of the 3xTg mouse model and hypothesized solution**

While the 3xTg model is currently one of the better rodent models of AD, it does not recapitulate the development of ERSR in neurons, as these mice (female) in our study are not reported to develop any detectable ERSR in the CNS (nor did they in our hand) (Mota et al., 2015). A study of the phosphorylated tau proteins indicates significant differences between AD patients and 3xTg mice. Specifically, N-terminal truncation, minimal high molecular weight tau smears, and slight hyperphosphorylation,
weaken the prion-like properties of the p-tau protein in the 3xTg mice (L. Li et al., 2019). The significant differences in P-tau formation and propagation may be critical in the pathology associated with the model and impact the development of ERSR. While these aspects of the pathology are not phenocopied in the 3xTg mice, data from chapter 2 suggest that the microbiome from an AD patient transplanted into the 3xTg mice could make the mouse model more closely reflect the human disease. Due to the extreme differences in lifespan, mice do not have the time to develop the same level of pathology and the FMT may ‘jump start’ the process. The data from chapter 3 provide strong evidence that the functional loss of butyrate is part of the pathogenesis, but there was no identified pathogenic metabolite, bacterial product, or pathobiont associated with the disease from the analysis. This in part may be due to differences seen in the type and extent of dysbiosis seen in AD patients, which once transplanted would make the 3xTg mouse model more likely to replicate the more pathogenic prion-like P-tau seen in AD patients. Another reason the FMT may be beneficial to the model is that in our animal housing facilities, mice are strictly managed with respect to contagion exposure and diet, which is vastly different from the day-to-day life of any given population and could contribute to disease severity.

**Evidence for oral supplementation of butyrate reaching the brain**

One of the critical findings from chapter 2, was that oral admission of TB led to increased histone acetylation in the CNS (Fig. 17). These data critically demonstrate not only that the loss of butyrate-producing bacteria leads to a decrease in acetylated histone of the brain but also that butyrate supplementation can prevent that deficit (Fig. 17). This discovery provides robust evidence that the SCFA butyrate is transported from the gut to the CNS. Future studies are planned to explore the differential gene expression as a result of oral supplementation of TB both in the 3xTg and FMT model.
One of the critical events noted is neurons, particularly in the dentate gyrus of the hippocampus, had significantly less acetylation as age increased (Fig. 17). Of particular importance is the fact that microglia do not express the typical butyrate receptor, free fatty acid receptor 2 (Erny et al., 2015). The data in chapter 3 and lack of free fatty acid receptor 2 on microglia suggest that the effect of butyrate likely affects another cell type, one of which is hypothesized to be neurons, that then affects microglia.

Hypothesis of how neuronal change in gene expression may affect the local environment

Neurons express proteins that aid in neuron-to-neuron communication but also neuron-to-glia communication (Sheridan & Murphy, 2013). One of the key neuron-to-glia signal molecules is fractalkine, which inhibits inflammatory activation of microglia, reducing MHC-II and IL-1β expression (Lyons et al., 2009). A key step in AD-related, protein-induced neuroinflammation is the activation of microglia (Calvo-Rodriguez et al., 2020; Dagher et al., 2015; Leng & Edison, 2021). As part of the disease, Aβ activates microglia through TLR4, initiating the inflammasome signaling cascade (Y. Liu, Y. Dai, et al., 2020). Microglial activation has been linked to or implicated in the development of oxidative stress and NFT propagation (Clayton et al., 2021; Colton et al., 2002; Stalder et al., 1999). Current work indicates that the loss of butyrate and increased Aβ plaques lead to increased microglia activation, while supplementation of tributyrin reduced that microglia activation (Fig. 14 A,B). While our study shows this to be a cause and effect, the mechanisms of how TB may be preventing microglial activation have not yet been elucidated. The increased transcriptional permissibility indicated by acetylation state in the neurons could lead to them expressing normal levels of fractalkine in the aged mice which will prevent the inflammatory activation of microglia even in the face of a TLR4 agonist (Lyons et al., 2009). Other studies indicate that fractalkine expression decreases with age and may be associated with age-associated neuroinflammation (Lyons et al., 2009). The reduction of fractalkine expression and the presence of the Aβ would lead to
microglia activation. In fact, it has been hypothesized that targeting the loss of fractalkine may be therapeutically effective in the treatment of AD patients (Duan et al., 2008). The prevention of microglial activation would also prevent the development of oxidative stress and NFT propagation, as seen in chapter 3 (Fig. 15 C-F). Thus, future studies will determine if the hypothesis that GM-derived butyrate plays a role in fractalkine expression is correct.

**Direct effects of butyrate on microglia**

Butyrate can have direct effects on microglia even though there are reports that the cell type does not express free fatty acid receptor 2 (Erny et al., 2015). Butyrate modulates the way that microglia respond to LPS and when pretreated with butyrate prevents the expression of cytokines (Huuskonen et al., 2004). In our model, the loss of butyrate production from the GM preceded significant increases in microglial activation (Fig. 14 A,B). These data suggest that the prolonged loss of butyrate increases the activation status of the microglia. The data support the idea that reversing this loss of butyrate with TB supplementation while in the presence of similar levels of plaque burden halted the progression of the disease. Our data support the claims that microglia play an important role in the pathogenesis of AD and have a potential to become pathogenic (Hansen, Hanson, & Sheng, 2018). Importantly, the activation status and capacity to tolerate pathogenic levels of Aβ is connected to butyrate produced in the GM. Supporting the importance of these data is the observance of a population of patients who, despite the relevant biological markers, do not develop AD. These patients are deemed non-demented with Alzheimer’s neuropathology, or NDAN (Arriagada et al., 1992; Bjorklund et al., 2012). Our data seem to help bridge this gap in the understanding of AD in that by supplementing butyrate at 6 months after Aβ production and propagation has begun, butyrate changed the response of microglia to the pro-inflammatory Aβ plaques.
Conclusion

The prevention of microglia activation did not create the environment necessary for the induction of oxidative stress and propagation of P-tau and therefore, halted the progression of the disease creating a NDAN-like population in our 3xTg model. This supports the concept that treating AD patients with butyrate and/or butyrate-producing bacteria would improve the functional symptoms typically associated with the disease is supported.

Chapter 4: identification of AR as a critical mediator of LPS-induced inflammation

LPS-induced inflammation is in part dependent on AR and its genetic ablation results in reduced inflammation. Specifically, ablation of AR successfully reduced the LPS (i.p.) driven infiltration in both the liver and brain (Fig. 19). ARKO mice also had a reduction in circulating cytokines and reduced the cytokines in the CNS. Finally, the cell culture work provides evidence that the inhibition of AR changes the inflammatory response to LPS. These data indicate that AR is a critical mediator in LPS-driven inflammation, and its inhibition blunts the inflammatory response.

Repurposing AR inhibitors as potential drugs for patients with diseases characterized by dysbiosis: Patients with AUD

The current data presented supports the idea to use AR inhibitors in the treatment of AUD and AD, as well as other diseases that are characterized by dysbiosis. Our laboratories have previously shown that AR inhibition had a positive impact on alcohol-induced ER stress through reducing fructose and uric acid (M. Wang et al., 2020). In the study described in chapter 2, the there is an elevation in ER stress, specifically in neurons in response to AH-FMT. These events that lead to an increase in ER stress could be linked to the metabolic changes associated with ethanol consumption that led to an increase in sorbitol, fructose, and uric acid. Specifically, uric acid was significantly elevated in the serum of ethanol-fed mice (M. Wang et al., 2020). Uric acid plays an
important role as an antioxidant (Becker, 1993). Moreover, in neurons, uric acid can have a protective effect against oxidative stress and can help protect neurons in reperfusion injuries (Ya et al., 2018). However, sustained elevated levels in uric acid are commonly seen in diabetics with neuropathy, and these changes affect the brain leading to an exaggerated response to cerebral ischemia (Srinivasan & Sharma, 2011). These data indicate that while uric acid has a critical function within the body and brain, it can reach pathologic levels where it no longer acts in a protective manner. Indeed, uric acid can lead to neurocognitive decline and inflammation, providing further evidence that maintaining a physiological level is critical to the health of the organism (Ya et al., 2018).

While ethanol does not have as strong an effect as reperfusion injuries, the increase in uric acid may play an important role in the ERSR in response to the AH-FMT and AF-AH-FMT. This would suggest that an inhibitor of AR may help alleviate the ERSR response in the CNS by reducing circulating uric acid to non-pathogenic levels. Future studies are warranted to determine if both uric acid and fructose are contributing to the pathogenesis in the FMT model of AUD and if uric acid plays an important and similar role in ethanol-induced neuroinflammation and neurodegeneration.

As discussed in chapter 4, the AR inhibitor could also positively impact the neutrophil infiltration in both the liver and brain of patients with AUD. All these data collectively show that the management of inflammation-related symptoms with an AR inhibitor could significantly impact the pathogenesis in alcohol-driven inflammation.

**Repurposing AR inhibitors as potential drugs for patients with diseases characterized by dysbiosis: Patients with AD**

Interestingly, AD patients have increases in glucose, sorbitol, and fructose in most regions on the brain (Xu et al., 2016). These data indicate that the increase in glucose is leading to not only glycolysis but also is being utilized in the polyol pathway.
As a product of the polyol pathway, fructose metabolism will result in ATP metabolized to ADP which is shuttled into the xanthine pathway forming uric acid. Although AR remains at similar levels in AD patients compared to controls, there is a decrease in the number of neurons indicating there is a higher AR to neuron ratio (Picklo, Olson, Markesbery, & Montine, 2001). The products of AR activation, fructose, and uric acid, may have an important role in the development of AD.

Uric acid and AR increase the concentrations of each other, where an increase in the expression of AR leads to increased uric acid and an increase in uric acid increases AR expression (Sanchez-Lozada et al., 2019; M. Wang et al., 2020). Uric acid, at normal levels acts as an antioxidant but at higher concentrations begins to induce oxidative stress (Sautin & Johnson, 2008). Another pathway through which AR mediates oxidative stress is through the polyol pathway (Srivastava et al., 2011). As a result of increased AR expression, glucose is shuttled into the polyol pathway leading to an increase in sorbitol and fructose. AR, the rate-limiting enzyme in the polyol pathway, requires the co-factor, reduced nicotinamide adenine dinucleotide phosphate (NADPH) which is oxidized to NADP+. NADPH, like uric acid, is an antioxidant which cells use to eliminate ROS (Tarafdar & Pula, 2018). Not only would the reduction of NADPH impair the capacity to process ROS, but the final product fructose also been reported to induce oxidative stress and increases lipid peroxidation in neurons in a model of traumatic brain injury (Agrawal et al., 2016). As described in chapter 3, both the 3xTg mice and AD patients develop oxidative stress (Fig. 14 C-F.). The toxic accumulation of Aβ has been linked to the development of oxidative stress in neurons (De Felice et al., 2007). The induction of oxidative stress not only disrupts the normal functions of neurons but also has been linked to furthering the production of Aβ (F. Li et al., 2004). The increase in oxidative stress will result in increased lipid peroxidation and could cause apoptosis (Kannan &
Jain, 2000). These data suggest that the targeting of oxidative stress could aid in the treatment of AD.

AR plays a role in the inflammatory response, particularly through the activation and translocation NF-κB. Data in chapter 4, as well as reported data, indicate that the inhibition of AR results in macrophages expressing different cytokines as well as enhancing their phagocytic capacity (Fig. 24) (Ramana, Willis, et al., 2006; M. Singh, Kapoor, McCracken, Hill, & Bhatnagar, 2017). Indeed, AR impacts phospholipase C and ultimately reduces NF-κB translocation into the nucleus (C. Wang et al., 2020). These data suggest that AR inhibition could be therapeutic in diseases associated with endotoxemia and dysbiosis. By increasing the capacity of the macrophages to phagocytize LPS and concomitantly not increase inflammatory cytokine expression, endotoxin tolerance may be reduced.

Repurposing AR inhibitor, Epalrestat, as a potential drug for patients with diseases characterized by dysbiosis: Patients with AUD

On addition to the effects in the CNS, ethanol is a powerful immunomodulator. Ethanol primes macrophages to have an exaggerated response to LPS (Gobejishvili, Barve, Joshi-Barve, & McClain, 2008). Along with these systemic effects, ethanol consumption can lead to liver disease, characterized by neutrophil infiltration, increased fat deposition and hepatocyte death. The stages of AUD progress from alcohol-induced fatty liver disease to alcoholic hepatitis and cirrhosis (Osna, Donohue, & Kharbanda, 2017). Moreover, there is enhanced chronic inflammation in patients with AUD. Data from chapter 4 indicate that LPS alone can drive neutrophil infiltration into the brain and liver (Fig. 19). Thus, the immune modulatory effects of the GM will be important, as a greater understanding of the gut-liver axis will have an increasing importance in the understanding and treatment of this disease. The genetic ablation of AR had a
significant impact on the capacity of LPS to drive neutrophil infiltration into the liver as well as reducing production of many cytokines (Fig. 19, 18). These data indicate that not only could AR inhibition aid in neurological issues related to ethanol consumption but could also be beneficial for those experiencing liver diseases. Due to the gut-liver-brain axis related to HE, treatment before developing the neurological pathology could protect the brain.

In the pathogenesis of AD, microglia activation has been implicated as an important component. In fact, activated microglia have been associated with P-tau propagation due to their response to Aβ (Clayton et al., 2021; Stalder et al., 1999). Data from chapter 4 show that the microglia in the CNS of AR null mice did not prevent the activation in response to LPS. However, cytokines levels from the brains of these mice indicate that both TNF-α and IL-1β were reduced when compared to the WT-LPS (Fig. 22). Since LPS and Aβ are TLR-4 receptor agonists, they drive similar inflammasome activation (Arioz et al., 2019; Y. Liu, Y. Dai, et al., 2020). This suggests that an AR inhibitor that can cross the BBB could reduce the cytokine expression by the microglia. Similarly, if the inhibitor increases the capability of microglia to phagocytize in the same way that it does with peripheral macrophages, that could also help reduce the Aβ burden in AD patients. Hence, inhibition of AR may prove to attenuate the inflammatory response caused by AD.

**Final Conclusions and future directions**

The development of ERSR in the PC due to ethanol consumption paired with the dysbiotic microbiome of Patients with AH merits deeper investigation. The fact that these cells did not express XBP1 like the other neurons examined, indicate these cells may undergo a different response. The lack of XBP1 suggest that the PCs are undergoing the integrated stress response while the other neurons are undergoing the
unfolded protein response. MRI studies of human patients indicate that the PCs shrink, and this phenotype may be a result of the ERSR/ISR activation (Andersen, 2004). Further studies will show how these cells are responding to the treatment conditions and seek to determine why these neurons respond differently than the hippocampal and prefrontal cortex neurons.

These data provide the basis for further experimentation aimed at combining several key factors. There is limited literature for the 3xTg AD mouse model is on whether ethanol affects the progression of the diseases. Since ethanol-induced dysbiosis is characterized by a loss of butyrate-producing bacteria, it should hasten the loss of butyrate-producing bacteria in the 3xTg mouse model, which would likely shorten the timeframe to the development of AD. The chronic liver damage ethanol feeding model used in chapter 2 is not suitable to study the effect of ethanol. Rather, a chronic binge model that replicates “weekend binge drinking” would be more relevant to the way young adults drink. The experiment would illustrate the role ethanol has on the familial inheritable version of AD.

The 3xTg mouse model is a good representation of early onset AD, but studies have shown that most of these patients do not have familial (inheritable) AD (Joshi, Ringman, Lee, Juarez, & Mendez, 2012). While the mouse models have been developed based on the familial genes related to AD, the GM is emerging as a major factor in the pathogenesis of AD (S. Liu, Gao, Zhu, Liu, & Zhang, 2020). In order to directly implicate the GM in the pathogenesis of AD the use of the novel FMT model in convention mice would help to elucidate the direct role of the GM by transplanting the GM from AD patients into these mice. The logistics and longevity of the transplantation would have to be prolonged to allow for the development of AD.
The combination of the work in chapters 2 and 3 with the inhibition of AR seen in chapter 4 may provide a therapeutic strategy that could positively affect the accumulation of ER stress, oxidative stress, and inflammation in both preclinical models and patients with AD and AUD. The evidence from these studies show that a targeted approach to address the functional changes due to dysbiosis has therapeutic potential. However, these benefits would require time to achieve the intended restoration of a normal functional microbiome. Therefore, SCFA supplementation, when appropriate, to manage long-term microbiome-driven effects, could be paired with an AR inhibitor in the treatment of some of the symptoms of AUD and AD. Through this method, ER stress, oxidative stress, and inflammation would be targeted by the AR inhibitor as well as addressing the underlying and driving problem arising from dysbiosis.
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ABBREVIATIONS

3xTg: triple transgenic mouse model of early-onset AD
4-HNE: 4-hydroxynonenal
ABO: Aβ oligomers
acrolein: 2-propanal
AD: Alzheimer's disease
AF: alcohol-fed
AH: Alcoholic hepatitis
AH-FMT: Fecal specimens from Patients with AH
APP: Amyloid precursor protein
ALD: Alcoholic liver disease
ALS: Amyotrophic lateral sclerosis
AR: Aldose reductase
ARKO: Aldose reductase knockout
ASF: Altered Schaedler Flora
ATF: activating transcription factor
AUD: Alcohol use disorder
Aβ: Amyloid-β
BBB: Blood brain barrier
BDNF: Brain derived neurotropic factor
CCL20: Chemokine (C-C motif) ligand 20
CCR6: Chemokine receptor 6
CHOP: C/EBP homologous protein
CNS: central nervous system
CSF: Cerebral spinal fluid
CX3CR1: fractalkine receptor
DAMP: Danger associated molecular pattern
DC: Dendritic cells
eIF2α: eukaryotic initiation factor 2 alpha
ER: Endoplasmic reticulum
ERSR: Endoplasmic reticulum stress response
F/B ratio: Ratio of Firmicutes to Bacteroidetes
FFAR2: Free fatty acid receptor 2
FMT: Fecal matter transplants
GALT: Gut-associated lymphoid tissue
GC: Glucocorticoids
GF: Germ-free
GFAP: Glial fibrillary acidic protein
GM: Gut microbiome
GRP78: Glucose-regulated protein 78
HD: Huntington's disease
HDAC: Histone deacetylases
HE: Hepatic encephalopathy
HMGB1: High mobility group box 1
HPA: Hypothalamic-pituitary-adrenal axis
HSR: Heat shock response
IACUC: Institutional Animal Care and Use committee
IBA-1: Ionized calcium-binding adapter molecule 1
IBC: Institutional Biosafety committee
IL: Interleukin
ISR: Integrated stress response
IMG: Integrated Microbial Genomes
IMM: indigenous mouse microbiome
KEGG: Kyoto Encyclopedia of Genes and Genomes
LPS: lipopolysaccharide
MAPT: microtubule associated protein tau
MCP: Monocyte chemoattractant protein
MELD: Model for end-stage liver disease
MIP: macrophage inflammatory protein
MS: Multiple sclerosis
NADPH: Reduced nicotinamide adenine dinucleotide phosphate
nFMT: healthy normal control subjects
NFT: Neurofibrillary tangles
NF-κb: nuclear factor kappa B
NORT: Novel object recognition test
OTUs: Operational taxonomic units
PAMP: Pathogen-associated-molecular-patterns
PERK: Protein kinase RNA-like ER kinase
PBS: phosphate buffered saline
PC: Purkinje cell
PCoA: Principal coordinates analysis
PD: Parkinson’s disease
PEG: polyethylene glycol
PF: pair-fed
PFA: Paraformaldehyde
PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PN: Proteostasis network
PS1: presenilin-1
P-tau: Phosphorylated tau
QIIME: Quantitative Insights into the Microbial Ecology
RA: Retinoic acid
ROS: Reactive oxygen species
SB: Staining buffer
SCFA: Short chain fatty acids
SEKDEL: GRP78 and GRP94
TB: tributyrin
TCR: T-cell receptor
Th: T helper
TJ: Tight junction
TLR: Toll-like receptor
UPR: Unfolded protein response
UPS: Ubiquitin proteasome system
Tregs: Regulatory T cell
WGS: whole genome shotgun
XBP1: X-box binding protein 1
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