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Indigenous gut microbes modulate neural cell state and neurodegenerative disease susceptibility

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Indigenous gut microbes modulate neural cell state and neurodegenerative disease susceptibility

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B.A., Colby College, 2019

Advisor: Timothy Sampson, Ph.D.

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Abstract

Indigenous gut microbes modulate neural cell state and neurodegenerative disease susceptibility
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The human gastrointestinal tract is home to trillions of microorganisms—collectively referred to as the gut microbiome—that maintain a symbiotic relationship with their host. This diverse community of microbes grows and changes as we do, with developmental, lifestyle, and environmental factors all shaping microbiome community structure. Increasing evidence suggests this relationship is bidirectional, with the microbiome also influencing a wide range of host physiological processes, including various aspects of neurological health. However, the ways in which the native microbiome concurrently impacts diverse brain cell types remain poorly understood. Therefore, this thesis begins by characterizing microbiome-dependent transcriptional changes across hippocampal cell types using single nucleus RNA sequencing (snRNA-seq) of wild-type germ-free (GF) mice, born and raised in a sterile environment. Simultaneous profiling of all major cell types allowed for direct comparison of transcriptional changes occurring within specific cell populations, identifying cell-type-specific and conserved microbiome-dependent transcriptional changes. This analysis highlighted an increase in adaptive immune and neurodegenerative disease-associated pathways across cell types in GF mice, highlighting a potential link between microbial signals and disease susceptibility. Therefore, to explore the sufficiency of specific indigenous microbes to mediate neuroimmune outcomes, wildtype GF mice were mono-colonized with select taxa associated with human neurological disease. RNA sequencing of brain myeloid cells from mice mono-colonized with *Escherichia coli, Clostridium celatum, Bacteroides thetaiotaomicron,* and *Lactobacillus johnsonii* each displayed their own unique phenotypes, highlighting species-specific effects of the microbiome on neuroinflammatory tone. One organism, *E. coli,* induced a unique adaptive immune and neurodegenerative disease-associated state, suggesting an increased disease susceptibility. SnRNA-seq of the hippocampus of *E. coli* mono-colonized mice demonstrated time and cell-type-dependent effects of *E. coli* on the brain, with changes in adaptive immune and neurodegenerative disease pathways occurring across cell types and time points. Further highlighting the importance of these transcriptional changes for disease outcomes, exposure of the 5xFAD beta-amyloidosis mouse model to *E. coli* resulted in exacerbated cognitive decline and amyloid pathology, demonstrating that this bacterium is sufficient to worsen AD-relevant outcomes. Together, these results emphasize the wide-reaching, species-specific, microbiome-dependent consequences on neurological functions, highlighting the capacity of specific microbes to modulate brain health and disease susceptibility.

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Chapter 1: Overview of the Gut Microbiome

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1.1 Abstract

The human gastrointestinal tract is home to trillions of microorganisms—collectively referred to as the gut microbiome—that maintain a symbiotic relationship with their host. This diverse community of microbes grows and changes as we do, with developmental, lifestyle, and environmental factors all shaping microbiome community structure. Increasing evidence suggests this relationship is bidirectional, with the microbiome also influencing host physiological processes. For example, changes in the gut microbiome have been shown to alter neurodevelopment and have lifelong effects on the brain and behavior. Age-related changes in gut microbiome composition have also been linked to inflammatory changes in the brain, perhaps increasing susceptibility to neurological disease. Indeed, associations between gut dysbiosis and many age-related neurological diseases—including Parkinson's disease, Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis—have been reported. Further, microbiome manipulation in animal models of disease highlights a potential role for the gut microbiome in disease development and progression. Although much remains unknown, these associations open up an exciting new world of therapeutic targets, potentially allowing for improved quality of life for a wide range of patient populations.

1.2 Introduction

Our bodies live in intimate association with a complex community of microbes: the microbiome. This consortium of microorganisms (inclusive of bacteria, archaea, fungi, and viruses) exists in a dynamic relationship with our bodies, and each other, as we age. Every environmentally-exposed surface—including our skin, oral cavity, and gastrointestinal (GI) tract—harbors a uniquely structured microbial community. These organisms have a distinct capacity to successfully inhabit each of these stringent niches, drawing on discrete carbon and energy sources, and resisting environmental and host-derived pressures (e.g. the immune response). In return, these microbial

symbionts provide critical metabolic and nutritional inputs, drive the development of our immune system, and perform a myriad of physiological functions throughout life.

While every exposed surface of our body is colonized by microbes, the microbiome of the GI tract is the largest and most complex. It is predicted that an equal number of bacteria per human cell exists within the gut microbiome(Sender, Fuchs et al. 2016). More impressively, current observations suggest an astounding ~3,500 unique species inhabit the human gut worldwide(Leviatan, Shoer et al. 2022), with each individual harboring ~100-500 unique species that are estimated to encode 100-fold more genes than the human genome(Qin, Li et al. 2010, Scepanovic, Hodel et al. 2019, Leviatan, Shoer et al. 2022). Within the GI tract, unique communities of microbes inhabit distinct niches. Biochemical, nutritional, and immunological gradients found throughout the gut create distinct environmental pressures that select for specific bacterial communities. For instance, the most proximal portion of the duodenum is environmentally characterized by an abundance of proteases, lipases, and bile salts, which are all necessary for appropriate digestion and absorption. The activity of these, in conjunction with acidic secretions from the gastric system, creates an environment where only a subset of microbes—typically fast-growing-facultative anaerobes—can fruitfully survive(Donaldson, Lee et al. 2016). More distally in the small intestine, these selective pressures decrease, and the overall bacterial load and species diversity increases. In the colon where water absorption occurs, the anaerobic environment creates prime conditions for the fermentation and production of methane, hydrogen, and carbon dioxide and selects for fermentative polysaccharide-degrading anaerobes, such as *Bacteroidaceae* and *Clostridia*(Donaldson, Lee et al. 2016).

Microenvironments also exist within each region of the small intestine and colon. The luminal environment—in the center of the GI tract—is highly anaerobic, only supporting the growth of anaerobic microbes. In contrast, the mucosal layer that creates a barrier between the gut lumen and epithelial tissue contains some residual oxygen content and facilitates the growth of oxygen-tolerant

organisms such as *Proteobacteria* and *Acinobacteria*(Albenberg, Esipova et al. 2014). Organisms that reside within this mucosa also have access to host-derived mucin glycans, a rich source of carbohydrates and protein. Bacteria such as *Akkermansia muciniphila* have been shown to break down mucin and are found in high abundance within the mucosal layer(Png, Lindén et al. 2010, Berry, Stecher et al. 2013). As epithelial cells slough away from the surface during cell turnover and luminal contents are mixed by peristalsis, organisms resident in each of these niches become intermingled into stool. Thus, while not a perfect representation of each niche separately, stool represents a reasonable—and technically feasible—platform to assess the many organisms found within the GI tract.

1.3 The gut microbiome through development and aging

The microbial composition of the GI tract is not static and continues to evolve throughout the lifespan. Each stage of development is associated with unique shifts in the microbial community, allowing for a bidirectional relationship between the microbiome and the developing body and brain (Figure 1.1).

The placental environment is largely thought to remain microbially sterile (with a few studies reporting possible in-utero microbial colonization(Jimenez, Marin et al. 2008, DiGiulio 2012, Aagaard, Ma et al. 2014, Zheng, Xiao et al. 2015, Collado, Rautava et al. 2016)), causing the developing fetus to remain un-colonized until birth(Lauder, Roche et al. 2016, Perez-Munoz, Arrieta et al. 2017, Briana, Papaevangelou et al. 2021). Even though the fetus may not make direct contact with living microbes, the indigenous maternal microbiome modulates the fetal environment through microbial metabolites, and via immune and metabolic signals that can cross (or signal through) the placental barrier. As such, changes in the maternal microbiome significantly impact fetal health and development. The maternal microbiome makes noticeable shifts in microbial composition that

coincide with key stages in gestation. During early stages of pregnancy, the maternal GI microbiome resembles that of a typical adult. However, during the third trimester, alpha diversity (the richness and evenness of the types of microbes present) decreases and the microbial community shifts to include more *Actinobacteria* and *Proteobacteria*(Koren, Goodrich et al. 2012). When given to germ-free mice, this pregnancy-associated GI microbiome induces metabolic changes that have the potential to shape neonatal developmental processes, including development of the nervous system(Koren, Goodrich et al. 2012).

The newborn's first exposure to external microbes varies considerably, depending on the mode of delivery. Infants born by vaginal delivery tend to have an initial microbiome composition that more closely resembles the maternal vaginal canal (enriched with *Lactobacillus, Prevotella,* and *Sneathia* spp.), whereas the microbiome of infants born by cesarian section has an increase in bacteria found on skin (such as *Staphylococcus, Corynebacterium*, and *Propionibacterium* spp.)(Dominguez-Bello, Costello et al. 2010). Although the differences between infants born by vaginal and cesarian section diminish with time(Chu, Ma et al. 2017, Hill, Lynch et al. 2017), these alterations in microbiome composition fall during a key critical window for neurological and immune development, with potential lifelong impacts. Unlike later stages of life when each environmental niche/body system has its own unique microbial community, the newborn microbiome begins relatively uniform across the entire body, with similar microbial composition on each body surface(Dominguez-Bello, Costello et al. 2010, Chu, Ma et al. 2017).

The infant microbiome is subsequently intimately shaped by early life diet, including the prebiotic and probiotic elements of breastmilk. Human milk oligosaccharides (HMOs)—the third most abundant component of breastmilk—promote the growth of beneficial microbes, including *Bifidobacterium* spp., which are uniquely adapted to ferment these indigestible complex glycans(Ward, Ninonuevo et al. 2006). HMOs also have antimicrobial activity against several harmful bacterial

strains, thus limiting potential infant infections(Ackerman, Doster et al. 2017, Ackerman, Craft et al. 2018, Chambers and Townsend 2020). In addition to shaping the existing infant microbial community, breastmilk also contains its own unique microbiome that is then passed on to the infant. In fact, during the first month of life, infants are estimated to receive 27.7% of their gut bacteria from breastmilk(Pannaraj, Li et al. 2017). It is, therefore, not surprising that the microbiome—and health outcomes—of breastfed infants are different from those who receive formula diets lacking in both these prebiotic and probiotic elements. Instead of microbiomes enriched with *Bifidobacterium* and *Lactobacillus* ssp., formula-fed infants display elevated levels of *Clostridium difficile*, *Granulicatella adiacens*, *Citrobacter spp*., *Enterobacter cloacae*, and *Bilophila wadsworthia*(Backhed, Roswall et al. 2015*)*.

Weaning and the introduction of solid food serves as an important trigger for continued diversification and transformation of the microbiome. Novel nutrients allow for an increase in alpha diversity as more types of bacterial taxa begin to thrive. This results in an overall increase in *Firmicutes* and *Bacteroidetes*—the dominant phyla throughout adulthood—and a subsequent decline in the previously dominant *Proteobacteria* and *Actinobacteria* (reviewed by (Milani, Duranti et al. 2017)). After around the age of three, the microbiome becomes more stable and less susceptible to perturbation, causing an overall decrease in beta diversity (variability in the composition of microbial taxa) as the microbiome composition begins to resemble that of a typical adult(Milani, Duranti et al. 2017). Although numerous lifestyle and environmental factors (discussed in the next section) can still induce subtle changes in overall community structure, larger and more long-lasting disturbances are needed to see an effect on the adult gut microbiome.

As individuals age, alpha diversity begins to decline, and the microbiome returns to a more unstable and malleable state(Claesson, Cusack et al. 2011). Aging-associated changes in hormone levels, metabolism, inflammation, GI physiology, and dietary/lifestyle factors disrupt the homeostasis of the microbiome, triggering widescale community restructuring. Unlike early life

changes that occur during more defined developmental windows, aging-associated microbiome shifts are far less predictable in timing and magnitude, depending on an individual's overall health and lifestyle(O'Toole and Jeffery 2015). Consequently, there is considerably more variability between studies characterizing the aged microbiome. In general, however, the elderly microbiome (65 and older) is characterized by a shift in the ratio of *Firmicutes* to *Bacteroidetes*, with the relative amount of *Bacteroidetes* increasing with age(Mariat, Firmesse et al. 2009, Claesson, Cusack et al. 2011, O'Toole and Jeffery 2015). Further insight into the microbiome in healthy aging may be gained through studies of centenarians and the unique microbiome composition associated with extreme aging and longevity. Although alpha diversity generally declines with age, centenarians tend to have increased alpha diversity compared with other less healthy elderly people(Kong, Hua et al. 2016). In addition, enrichment of *Akkermansia* is often evident, suggesting a contribution to longevity(Biagi, Franceschi et al. 2016, Kong, Hua et al. 2016) or a consequence of a highly aged intestinal environment.

1.4 Perturbations to the gut microbiome

In addition to age-related changes in microbiome composition, many lifestyle and environmental factors have been shown to modulate the microbial community within the gut. As stated above, the adult microbiome is generally considered to be remarkably stable, undergoing relatively minimal changes in the absence of overt perturbation(Faith, Guruge et al. 2013). The overall resilience of the gut microbiome is supported at both the microbial and host level. The highly diverse and interconnected community structure of the microbiome resists colonization by novel microbes and supports the overall community resilience(Fassarella, Blaak et al. 2021). Similarly, stable selective pressures from the side of the host support the growth, and regrowth of specific taxa after minor perturbations(Fassarella, Blaak et al. 2021). As such, the microbiome remains in a state of dynamic equilibrium in which minor lifestyle and environmental changes cause only transient alterations to

the overall community structure. However, more drastic or sustained selective pressures can represent tipping points that trigger widespread community restructuring(Fassarella, Blaak et al. 2021).

Perhaps the most obvious and critical factor influencing the microbiome throughout the lifespan is diet. By modulating the nutrients present in the GI tract, one can support or inhibit the growth of specific microbes within this ecosystem, causing dramatic shifts in the microbial community. These changes can occur on both short- and long-term timescales, depending on a person's dietary habits. For example, a sudden switch from a plant-based diet to an animal-based diet triggers substantial changes in microbiome composition within only one day(David, Maurice et al. 2014). Specifically, initiating an animal-based diet increased levels of bile-tolerant *Alistipes, Biophilia,* and *Bacteroides* while decreasing the levels of bacteria that metabolize plant polysaccharides, such as *Roseburia, Eubacterium rectale,* and *Ruminococcus bromii*(David, Maurice et al. 2014). Although brief dietary changes such as this do not produce lasting effects on microbiome composition,(David, Maurice et al. 2014) long-term dietary patterns can exert a sustained effect on the microbiome. A persistent high-fat diet, as one example, is associated with a unique microbiome composition in murine models that is generally characterized by increased *Firmicutes* and *Proteobacteria* with decreased *Bacteroidetes*(Hildebrandt, Hoffmann et al. 2009, Carmody, Gerber et al. 2015). In contrast, a Mediterranean diet (high in whole grains, nuts, legumes, fruits, and vegetables) is associated with an increase in *Bacteroides* and *Clostridium* and reductions in *Proteobacteria* and *Bacillaceae*(Marlow, Ellett et al. 2013).

Stress is another environmental factor that is known to influence the composition of the gut microbiome. Physiological changes associated with stress—including altered hypothalamic-pituitary-adrenal axis (HPA-axis) reactivity, GI motility, and immune activation—modify the intestinal environment and shape the microbial community. Prenatal stress can have a particularly significant impact on overall microbiome composition due to the increased malleability of the microbiome

during this developmental window (see the section on development and aging above). As such, gut dysbiosis associated with prenatal stress has been reported in both humans and animal models(Golubeva, Crampton et al. 2015, Zijlmans, Korpela et al. 2015, Gur, Shay et al. 2017). Infants of mothers who experienced high levels of stress throughout pregnancy display higher levels of bacterial taxa such as *Escherichia, Serratia,* and *Enterobacter* and lower levels of lactic acid bacteria (such as *Lactobacillus, Lactococcus, Aerococcus*) and *Bifidobacteria*(Zijlmans, Korpela et al. 2015). The effects of prenatal stress on the microbiome can be lifelong, with prenatally stressed rodents showing altered microbiome composition into adulthood(Golubeva, Crampton et al. 2015, Gur, Shay et al. 2017). Changes in the gut microbiome are similarly associated with stress levels during adulthood (Knowles, Nelson et al. 2008, Bailey, Dowd et al. 2011, Galley, Yu et al. 2014, Bharwani, Mian et al. 2016, Gautam, Kumar et al. 2018). For example, levels of lactic acid bacteria have been shown to decline during periods of high stress, such as during college exams(Knowles, Nelson et al. 2008).

A substantial number of studies have shown that exercise modifies the gut microbiome in both animal models and people(Mailing, Allen et al. 2019). Like many microbiome investigations, these studies often show quite disparate results potentially attributable to differences in experimental design, such as the type of exercise, dietary controls, and the subject's body mass index(Mailing, Allen et al. 2019). One of the more consistent trends, however, is an increase in bacteria that produce butyrate(Matsumoto, Inoue et al. 2008, Evans, LePard et al. 2014, Estaki, Pither et al. 2016, Bressa, Bailen-Andrino et al. 2017)—a short-chain fatty acid produced by bacterial fermentation of dietary fiber that has known effects on GI function, immunity, and the brain(Tan, McKenzie et al. 2014, Dalile, Van Oudenhove et al. 2019). One study, however, reported that this too may vary, based on body mass index, with an increase in butyrate-producing *Faecalibacterium* in lean participants but a decrease in obese participants(Allen, Mailing et al. 2018).

Specifically designed to target bacteria, antibiotic treatment also may cause rapid and drastic changes in overall microbiome composition. Although antibiotic treatment is an effective way to eliminate many significant bacterial pathogens, it also has the potential to have long-term impacts on the overall health of the microbiome. The consequences of antibiotic use vary, depending on the antibiotic type, dosage, and treatment duration, as well as an individual's starting microbiome composition and diet. Antibiotic treatment triggers a rapid depletion of many bacterial taxa—likely eliminating some species from the ecosystem entirely—and provides novel niches for antibiotic-resistant bacterial blooms(Schwartz, Langdon et al. 2020). While most microbes quickly regrow after the cession of antibiotic treatment and the overall microbiome composition re-stabilizes, numerous studies have demonstrated that an individual's microbiome composition may never fully recover to its pre-antibiotic state(Jernberg, Lofmark et al. 2007, Dethlefsen, Huse et al. 2008, Jakobsson, Jernberg et al. 2010, Dethlefsen and Relman 2011). This is especially true in people who are vulnerable due to other lifestyle and environmental factors. For example, antibiotic treatment is particularly disruptive during times when the microbiome is already unstable, such as early in the lifespan and during ageing(Schwartz, Langdon et al. 2020). In addition to changing the overall microbial composition, antibiotics modulate the activity and metabolism of microbes that remain present within the GI tract, altering their effects on the human body(Ferrer, Mendez-Garcia et al. 2017).

Many other factors shape the composition of the gut microbiome, including genetics, environmental exposures, sleep, and medication use(Cryan, O'Riordan et al. 2019). Together, these lifestyle factors all act in concert to define the pressures that select an individual's unique microbiome composition. As such, it's not surprising that many human microbiome studies struggle to account for these individual differences. This leads to challenges in the interpretation of correlational studies and difficulty generalizing results from one study to the next. Careful and

informed experiential design, representative controls, and large sample sizes are essential to mitigate these challenges. For example, there is a growing push towards the use of age matched-household controls in human microbiome studies in an attempt to limit confounding effects of environmental, physiological, and lifestyle factors on study results.

1.5 Contributions of the gut microbiome to brain health throughout the lifespan

The gut microbiome is poised to have a critical influence on numerous aspects of neurological development throughout the lifespan. The intimate association between the microbial community and body systems allows for dynamic interactions across development. In addition, the microbiome has a significant capacity for both metabolic and immune modulation, allowing it to shape numerous developmental processes. Using gnotobiotic experimental models, combined with detailed profiling of human-derived gut microbiomes across various disorders, the field is beginning to identify some of these key contributions of the gut microbiome to neurological health.

Starting during gestation, experimental studies using mouse models have linked maternal microbiome composition to several important outcomes within the developing fetus. For example, embryos derived from germ-free mice (born and raised in a sterile environment) display significantly increased blood-brain-barrier (BBB) permeability, corresponding with altered tight junction protein profiles in their brains(Braniste, Al-Asmakh et al. 2014). Germ-free murine embryos also display immature microglia phenotypes (Thion, Low et al. 2018) and diminished thalamocortical axon growth leading to deficits in sensory-motor behaviors later in life(Vuong, Pronovost et al. 2020). Although being germ-free is a highly artificial condition, these data highlight the presence of critical inputs from the microbiome to the developing brain. More coercively, it suggests that maternal microbiome-derived signals may predispose developing offspring to subsequent neurological deficits. For instance, increased BBB permeability may induce a shift in signals (e.g., small molecule

metabolites and cytokines) received within the brain parenchyma during this critical developmental window.

Although humans are not raised germ-free, many infants are exposed to antibiotic treatment either during gestation or shortly after birth, which can disrupt the microbial signals they receive. Epidemiological studies have linked this early-life antibiotic exposure to a wide range of negative health outcomes(Aversa, Atkinson et al. 2021). In addition, perinatal administration of antibiotics has been linked to behavioral changes in rodents, including hypoactivity and alterations to anxiety-like and social behaviors, often phenocopying germ-free animals(Vuong, Yano et al. 2017). Thus, not only is an intact microbiome required for many of these developmental aspects, but a complex and highly diverse microbiome is necessary.

Some impacts of the microbiome on neurological function appear following perturbation post-development and in adulthood. One prime example is the necessity of gut microbiome-derived signals for the development and continued maintenance of fully mature microglia. Germ-free or antibiotic-treated mice display microglia with an immature phenotype, less capable of mounting inflammatory responses and defense against pathogens(Erny, Hrabe de Angelis et al. 2015, Matcovitch-Natan, Winter et al. 2016, Thion, Low et al. 2018). Exposure to intact microbiomes or to the microbial metabolites, short-chain fatty acids (SCFAs), rescues these effects within only a few weeks, suggesting that the microbiome can modulate cellular activities in the brain actively in adulthood(Erny, Hrabe de Angelis et al. 2015). The gut microbiome also influences levels of important signaling molecules within the brain into adulthood. For example, germ-free and antibiotic-treated mice have altered brain derived neurotrophic factor (BDNF)(Bercik, Denou et al. 2011, Neufeld, Kang et al. 2011) (a protein involved in promoting neuronal survival and plasticity) as well as changes in several key neurotransmitters and their receptors(Diaz Heijtz, Wang et al. 2011, Neufeld, Kang et al. 2011, Clarke, Grenham et al. 2013).

As mammals age, circulating and tissue-resident levels of pro-inflammatory cytokines begin to basally increase, including within the brain. This process—often termed inflammaging—increases one's risk of age-related neurological disease(Zuo, Prather et al. 2019). These age-related changes in inflammatory state are accompanied by distinct changes in gut microbiome composition that may contribute to immunosenescence. In *Drosophila melanogaster,* for example, these microbiome changes predate and predict age-related intestinal barrier dysfunction, immune activation, and mortality(Clark, Salazar et al. 2015). Further, some experimental models have demonstrated that an aged microbiome is sufficient to induce some aspects of inflammaging in younger animals(Fransen, van Beek et al. 2017, Thevaranjan, Puchta et al. 2017). On the other hand, recolonization of older animals with microbiota from younger animals has the opposite effect, decreasing inflammation and improving longevity(Smith, Willemsen et al. 2017, Barcena, Valdes-Mas et al. 2019). Given that both aging and inflammation are major risk factors for neurodegenerative diseases, there is growing interest in how the aging microbiome may contribute to disease development.

1.6 Associations between neurological diseases and gut microbiome composition

With mounting evidence that the gut microbiome can shape a wide range of neurological functions, there is growing interest in the potential role of the gut microbiome in the pathophysiology of neurological diseases. As such, an increasing number of studies have attempted to characterize the gut microbiome composition associated with distinct patient populations to identify potential disease-modifying microbes. Compelling associations have begun to appear between the composition of the gut microbiome and a number of disease states including Parkinson's disease (PD), Alzheimer's disease (AD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) (Figure 1.2)(Fang, Kazmi et al. 2020). These, and others, are discussed in-depth in later reviews in this monograph.

Of the neurogenerative diseases, PD has some of the strongest associations with gut dysbiosis. Although typically considered a motor disorder, PD is also characterized by GI symptoms that can predate motor deficits by decades(Kalia and Lang 2015). Evidence of alpha-synuclein pathology in the gut suggests that in some PD patients, PD pathology may originate in the GI tract(Braak, de Vos et al. 2006). Changes in the gut microbiome of PD patients were first reported in 2015, highlighting the association between the gut microbiome and disease outcomes(Keshavarzian, Green et al. 2015, Scheperjans, Aho et al. 2015). Clinical studies characterizing the gut microbiota of PD patients have faced replication challenges due to variability from methodological differences, small sample sizes, and confounding variables. However, recent meta-analyses and larger-scale studies have begun to identify some consistent patterns(Tan, Lim et al. 2022). Of these, the most consistent finding is an increase in the mucin-degrading bacteria *Akkermansia*(Nishiwaki, Ito et al. 2020, Romano, Savva et al. 2021, Toh, Chong et al. 2022). Other consistent trends include an increase in *Bifidobacterium* and *Lactobacillus*(Romano, Savva et al. 2021, Wallen, Demirkan et al. 2022) and reductions in *Lachnospiraceae*,(Nishiwaki, Ito et al. 2020, Romano, Savva et al. 2021) *Faecalibacterium,*(Nishiwaki, Ito et al. 2020, Romano, Savva et al. 2021, Wallen, Demirkan et al. 2022) and *Roseburia*(Nishiwaki, Ito et al. 2020, Toh, Chong et al. 2022, Wallen, Demirkan et al. 2022).

As with PD, increasing evidence suggests that AD pathophysiology may be shaped by changes in the gut microbiome(Chandra, Sisodia et al. 2023). Although numerous smaller studies have been published attempting to characterize the AD microbiome, few metanalyses or large-scale studies have been performed. Consequently, the characteristics of the AD-associated microbiome—or if there even is a specific composition associated with AD—remains unclear. Despite these challenges, the few published studies identify some apparent compositional differences in those with AD. AD patients show a reduction in overall phylogenic diversity and a microbiome composition that is distinct from healthy controls(Vogt, Kerby et al. 2017, Liu, Wu et al. 2019, Ling, Zhu et al. 2020,

Hung, Chang et al. 2022). As in PD, several studies have demonstrated that AD patients have an increase in *Bifidobacterium* and the phylum *Actinobacteria* in which it resides(Zhuang, Shen et al. 2018, Ling, Zhu et al. 2020, Hung, Chang et al. 2022). In fact, *Bifidobacterium* was found to be positively correlated with worsened symptom severity(Ling, Zhu et al. 2020). However, another prominent study found a significant decrease in *Bifidobacterium* in AD patients that was correlated with worsened amyloid and tau CSF biomarkers(Vogt, Kerby et al. 2017), an example of significant discrepancies remaining in this field. Another trend that has appeared in several studies is an increase in *Escherichia/Shigella* and their phylum *Proteobacteria*(Cattaneo, Cattane et al. 2017, Liu, Wu et al. 2019). Levels of *Escherichia/Shigella* were positively correlated with serum pro-inflammatory cytokines in AD patients, suggesting a possible contribution of these bacteria to pathologic neuroinflammation(Cattaneo, Cattane et al. 2017). The family *Bacteroides* and phylum *Bacteroidetes* also appear different in AD, although the direction of change varies by geographical location(Vogt, Kerby et al. 2017, Zhuang, Shen et al. 2018, Hung, Chang et al. 2022). Finally, decreases in *Clostridium* have also been reported in several studies(Vogt, Kerby et al. 2017, Hung, Chang et al. 2022).

Although less researched than either PD or AD, growing evidence suggests that MS is also associated with an altered gut microbiome compared with healthy individuals(Correale, Hohlfeld et al. 2022). As in PD, MS patients have increased levels of *Akkermansia*(Jangi, Gandhi et al. 2016, Berer, Gerdes et al. 2017, Cekanaviciute, Yoo et al. 2017) and decreased levels of *Faecalibacterium*(Cantarel, Waubant et al. 2015, Miyake, Kim et al. 2015, Ling, Cheng et al. 2020). In fact, one study found a negative correlation between levels of *Faecalibacterium* and pro-inflammatory cytokines such as TNF(Ling, Cheng et al. 2020). MS patients have also been found to have decreased *Parabacteroides*(Chen, Chia et al. 2016, Cekanaviciute, Yoo et al. 2017), Butyricicoccus(Jangi, Gandhi et al. 2016, Ling, Cheng et al. 2020), and *Prevotella*(Miyake, Kim et al. 2015, Chen, Chia et al.

2016) and associated species. Of note, medication use significantly alters the microbiome composition of MS patients, highlighting the importance of controlling for such variables in these studies(Cantarel, Waubant et al. 2015, Jangi, Gandhi et al. 2016, Berer, Gerdes et al. 2017).

Finally, a few preliminary studies (all with sample sizes under 40) have hinted at possible changes in gut microbiome composition associated with ALS, but no generalizable trends have been established(Fang, Wang et al. 2016, Blacher, Bashiardes et al. 2019, Zhai, Zheng et al. 2019, Zeng, Shen et al. 2020). For example, the largest and most well-controlled study (with 37 ALS patients and 29 healthy family members) by Blacher et al. identified a distinct microbiome associated with ALS, with marginally significant increases in *Anaerostipes hadrus, Bacteroidales bacterium*, and *Bifidobacterium pseudocatenulatum* and marginally significant decreases in *Clostridium leptum* and *Escherichia coli*(Blacher, Bashiardes et al. 2019). In support of the positive association of *Anaerostipes hadrus* with ALS, another study similarly demonstrated an increase in this type of bacteria at the family (*Lachnospiraceae*) and genus level (*Anaerostipes*)(Fang, Wang et al. 2016). However, this is one of the few associations that has been replicated, highlighting the critical need for further observations. In fact, another study investigating the microbiome of ALS patients failed to identify significant differences in microbiome composition at all(Brenner, Hiergeist et al. 2018).

Although correlations between neurological diseases and microbiome composition are compelling, care must be taken in interpreting human disease-microbiome association studies to not equate associations (no matter how strong) with a microbiome contribution to disease. Many neurological diseases likely have impacts on the intestinal environment (covered in depth in other reviews in this monograph) that may shape microbiome community structure. For example, alterations to gut motility, intestinal permeability, inflammation, and mucin production may all select for specific bacterial types. In addition, dietary changes due to mood or motor control may also result in microbiome compositional changes. Indeed, a prominent example of how dietary changes

may mediate microbiome associations with neurological outcomes was a demonstration that the majority of microbiome changes associated with autism spectrum disorder (ASD) were more highly associated with restrictive and repetitive dietary habits, rather than the disorder itself (Yap, Henders et al. 2021). However, although this study serves as an example of how a disease may give rise to an altered microbiome (through dietary restrictions for instance), it does not prove that disease-associated microbiomes are merely epiphenomena. No matter the cause, changes in gut microbiome composition and function can modulate metabolic and immunologic profiles that are critical determinants of overall disease progression. Thus, to truly understand the consequences of discrete microbiome compositions on disease etiology, pathology, and progression, it is important to complement human association studies with more controlled experiments in animal models that can determine both the time course and consequences of dysbiosis.

1.7 Experimental impacts of the microbiome on neurological disease

To further understand the relationships between the gut microbiome and neurological diseases, researchers have turned to animal models that allow for direct manipulation of the gut microbiome. As such, increasing evidence suggests that disease-associated microbes can directly modulate disease outcomes in models of many neurological diseases including PD, AD, MS, and ALS.

As with the human data, the most substantial evidence for the role of the gut microbiome in neurological disease is seen within PD mouse models. Germ-free status or antibiotic treatment of alpha-synuclein overexpressing mice reduces model-dependent behavioral impairments and pathologies, suggesting a contribution of the microbiome to PD(Sampson, Debelius et al. 2016). Conversely, reconstituting intact human microbiomes derived from PD patients exacerbates these outcomes(Sampson, Debelius et al. 2016), suggesting that specific microbes found within the PD patient microbiome may act pathologically. As such, several studies have attempted to identify

specific PD-associated bacterial species that modulate disease outcomes. For example, studies have shown that *Proteus mirabilis*(Choi, Kim et al. 2018) and *Escherichia coli*(Chen, Stribinskis et al. 2016, Sampson, Challis et al. 2020) are both sufficient to exacerbate motor impairments in PD mouse models. In addition, both of these bacteria exacerbate inflammation and alpha-synuclein pathology in the colon and brain, suggesting a direct contribution to inflammatory and pathological processes(Chen, Stribinskis et al. 2016, Choi, Kim et al. 2018, Sampson, Challis et al. 2020). In parallel with these more subtle inflammatory insults, robust intestinal inflammation triggered by a transient bacterial infection(Matheoud, Cannon et al. 2019) or dextran sodium sulphate(Kishimoto, Zhu et al. 2019) has also been shown to exacerbate disease outcomes in PD mouse models, including the conversion to parkinsonism(Matheoud, Cannon et al. 2019).

Similar to PD, increasing evidence suggests that AD outcomes may be modulated by the gut microbiome. AD mouse models treated with high dose antibiotics(Minter, Zhang et al. 2016, Minter, Hinterleitner et al. 2017, Dodiya, Kuntz et al. 2019, Dodiya, Frith et al. 2020, Mezö, Dokalis et al. 2020, Dodiya, Lutz et al. 2022, Seo, O'Donnell et al. 2023) or raised in a germ-free environment(Harach, Marungruang et al. 2017, Mezö, Dokalis et al. 2020, Seo, O'Donnell et al. 2023) display significant reductions in amyloid and tau pathology, neurodegeneration, and neuroinflammation, highlighting the contribution of microbes to AD outcomes**.** Furthermore, the overall microbial composition that occurs in AD mouse models may play a role in the development of pathology. For example, colonization of antibiotic-treated or germ-free APP/PSEN1 mice with the microbiota of dysbiotic APP/PSEN1 mice exacerbates AD-like pathology(Harach, Marungruang et al. 2017, Dodiya, Kuntz et al. 2019) while colonization with healthy wild-type microbiota induces considerably less amyloid pathology(Harach, Marungruang et al. 2017). In addition, transfer of healthy wild-type microbiota to dysbiotic AD mouse models improves disease outcomes(Sun, Xu et al. 2019, Kim, Kim et al. 2020) whereas transfer of dysbiotic AD microbiota to wild-type mice

induces cognitive impairment and neuroinflammation(Kim, Jeon et al. 2021).

As with PD and AD, a growing body of evidence supports the role of the gut microbiome in the etiology of MS. One of the most common mouse models of MS is induced by inoculating mice with antigens of myelin oligodendrocyte glycoprotein, which triggers an autoimmune response called experimental autoimmune encephalomyelitis (EAE), which is similar (though not identical) to the human condition. Mice with an intact microbiome typically develop EAE within two weeks of inoculation, but germ-free and antibiotic-treated mice show significant attenuation of neuroinflammation and EAE symptoms, and many never develop EAE at all(Yokote, Miyake et al. 2008, Ochoa-Reparaz, Mielcarz et al. 2009, Berer, Mues et al. 2011, Lee, Menezes et al. 2011). The necessity of the gut microbiome for the full EAE presentation suggests that microbes contribute to the development of this autoimmune response. As with PD, colonization of germ-free mice with the microbiota from MS patients results in exacerbated EAE compared with mice colonized with fecal samples from healthy controls, highlighting the particularly detrimental effects of the MS-associated microbiome(Berer, Gerdes et al. 2017, Cekanaviciute, Yoo et al. 2017). Attempts to identify individual bacteria that are capable of modulating EAE outcomes have demonstrated that segmented filamentous bacteria (SFB) is sufficient to increase EAE susceptibility(Lee, Menezes et al. 2011), whereas *Bacteroides fragilis* polysaccharide A is protective(Ochoa-Reparaz, Mielcarz et al. 2010).

Unlike the other neurological diseases discussed herein, evidence from mouse models of ALS demonstrates a more protective role of the gut microbiome. For example, antibiotic treatment of the *Sod1*-Tg mouse model was found to *exacerbate*, rather than improve, motor symptoms in this model of ALS(Blacher, Bashiardes et al. 2019). Furthermore, deriving *Sod1*-Tg mice germ-free resulted in high mortality rates, suggesting that the microbiome may be protective in this model. Attempts to identify specific disease-relevant bacteria showed that *Ruminococcus torques* and *Parabacteroides distasonis* exacerbated disease outcomes and *Akkermansia muciniphila* was protective in this model(Blacher,

Bashiardes et al. 2019).

Together, preclinical data provide strong support for the potential role of the gut microbiome in neurological disease. However, whether these results will translate to human patients remains unknown. Although animal models often recapitulate key characteristics of the human condition, they fall short of truly replicating all elements of the disease pathophysiology. As such, preclinical data must be paired with well-controlled longitudinal microbiome association studies and clinical trials in order to fully understand the contribution of the gut microbiome to disease.

1.8 Microbiome-based therapeutics

With growing evidence supporting the capacity of the gut microbiome to modify neurological outcomes, there is increasing enthusiasm for the development of microbiome-based therapeutics. The gut microbiome could potentially serve as a relatively low-risk and easily modifiable treatment target for a wide range of neurological conditions. To date, there are three primary microbiome-based therapeutic options, each with their own advantages and disadvantages: 1) fecal microbiota transplant (FMT), 2) probiotics, and 3) prebiotics. Each of these treatment approaches has been utilized with varied success in both preclinical and clinical settings to treat a wide range of disease conditions.

With our currently limited understanding of the causal relationships between individual bacteria and disease outcomes, FMT—in which patients are given a complex microbial community derived from the fecal sample of a healthy donor—provides a promising possibility for the immediate implementation of a microbiome-based treatment. FMT alleviates the need for researchers to identify individual disease-modifying microbes (as is necessary for probiotic approaches), providing an accelerated approach to treating the millions of people currently suffering from neurological diseases. Furthermore, results from preclinical models of neurological disease, including PD(Sun,

Zhu et al. 2018, Zhao, Ning et al. 2021, Zhong, Chen et al. 2021), AD(Sun, Xu et al. 2019, Kim, Kim et al. 2020), and MS(Li, Wei et al. 2020), suggest that FMT may be effective in modifying disease outcomes. In addition, several small-scale studies point to the potential long-term benefits of FMT in those with PD(Huang, Xu et al. 2019, Xue, Yang et al. 2020, Kuai, Yao et al. 2021, Segal, Zlotnik et al. 2021), AD(Hazan 2020, Park, Lee et al. 2021), MS(Borody, Leis et al. 2011, Makkawi, Camara-Lemarroy et al. 2018, Engen, Zaferiou et al. 2020), and ALS(Lu, Wen et al. 2022). In order to truly determine the safety and efficacy of FMT for the treatment of neurological conditions, large-scale randomized, double-blind, placebo-controlled (or autologous FMT-controlled) clinical trials must be conducted. In response to this need, several trials are ongoing for PD (ClinicalTrials.gov # NCT05204641, NCT03808389, NCT04854291) and ALS (ClinicalTrials.gov # NCT03766321), but others are needed to explore the safety and efficacy in the context of other neurological diseases.

FMT allows for rapid implementation in the clinic before a complete understanding of the effects of individual microbes on disease outcomes is established, but this less targeted approach also opens the possibility of inadvertently introducing pathogenic microbes that may be detrimental to patients or interact with comorbidities or genotypes in unexpected ways. The potential risks of FMT have prompted many researchers to turn to more targeted approaches in which specific beneficial microbes (i.e. probiotics) are given to patients. To this effect, there has been great interest in using probiotics—primarily consisting of various *Lactobacillus* and *Bifidobacterium* species—for the treatment of neurological conditions in both preclinical and clinical studies. While more targeted, these approaches still come with the caveat that we are still early in our understanding of how these organisms precisely interact with us and other microbes in the community. Further, on their own, probiotics are not generally able to colonize an intact adult microbiome(Han, Lu et al. 2021). Although this does not negate the potential efficacy of hit-and-run type influences on the microbial community, it does suggest consistent dosing may be necessary for long-term effects.

Where FMT and probiotics facilitate the growth of beneficial microbes by providing a direct supplement of the desired living organisms, prebiotics take an alternative approach, instead providing the nutrients to support the growth and beneficial functions of microbes already present in the gut. Prebiotic supplementation has been shown to modulate gut microbiome composition and impact several neurological conditions. For example, treatment of alpha-synuclein overexpressing mice with a prebiotic high-fiber diet remodels the gut microbiome and attenuates motor deficits, alpha-synuclein pathology, and neuroinflammation(Abdel-Haq, Schlachetzki et al. 2022). Other dietary changes, such as adherence to a Mediterranean diet, which is known to promote the growth of beneficial microbes, can also modulate neurological disease outcomes. For example, switching to a Mediterranean diet altered the gut microbiome composition of patients with mild cognitive impairment and improved AD biomarkers in their cerebrospinal fluid(Nagpal, Neth et al. 2019).

Just as the gut microbiome is known to be modulated by a wide range of factors, the efficacy of microbiome-based treatment approaches is similarly shaped by lifestyle and genetic factors. For microbiome-based treatments to take hold, the intestinal environment needs to provide the correct conditions for beneficial microbes to thrive and outcompete potentially detrimental microbes. As such, a combination of FMT / probiotic treatments with additional therapeutic approaches such as prebiotics or lifestyle changes that facilitate the growth of beneficial microbes may hold the key to clinical efficacy.

1.9 Future outlooks

The gut microbiome has evolved to co-exist in a symbiotic relationship with the host throughout the lifespan. Just as host lifestyle and physiological processes shape the gut microbiome, growing evidence suggests that the gut microbiome shapes host health and development. As such, a better understanding of factors that modulate the health of the gut microbiome may hold the key to

understanding—and treating—many human diseases. Insight from longitudinal studies and animal models has begun to tease apart the intricate relationships between the gut microbiome and human health. With this comes growing evidence for the role of the microbiome in neurological processes, including neurodevelopment and neurological disease.

Despite growing evidence for the role of the gut microbiome in shaping neurological outcomes, much remains unknown. Although animal models highlight a potential contribution of the gut microbiome to neurological disease, it is still unknown whether similar processes may be occurring in humans. The lack of well-powered longitudinal studies has made it difficult to determine the potential etiological role of the gut microbiome in human disease. Most human studies to date have assessed microbiome composition after disease diagnosis, making it impossible to determine when dysbiosis may have developed. This leads to challenges in understanding the role of the microbiome in disease etiology, since it is unclear whether the alterations in the microbiome trigger disease onset, contribute to progression, or merely co-occur with disease. Microbiome-based treatment approaches are also limited by a lack of mechanistic insight into the specific microbes and signaling pathways involved in health and disease. As such, many clinicians are understandably hesitant to implement microbiome-based treatments at this time.

Although most gut microbiome research to date focuses solely on bacteria, this is merely scraping the surface of the entire gut microbiome. In order to truly understand the contribution of the gut microbiome to host health, one needs to consider the potential role of all the different microbial types (including bacteria, but also archaea, fungi, and viruses). For example, there is increasing interest in the ability of viruses and fungi to modulate physiological outcomes and overall microbiome health and community structure(Virgin 2014, Forbes, Bernstein et al. 2019, Santiago-Rodriguez and Hollister 2019).

In addition, microbial genes may act in combination with host genes to determine genetic

disease risk factors. For example, an association between PD risk genes and intestinal microbiome composition has been reported(Wallen, Stone et al. 2021). As such, there is a growing push across many realms of biomedical science to view the human body not as a single, isolated organism, but instead as a "holobiont" consisting of both host and microbial inputs. By increasing our understanding of how the holobiont develops and responds throughout health, aging, and disease, we will gain insight into how to better prevent, detect, and treat numerous neurological diseases.

1.10 Figures

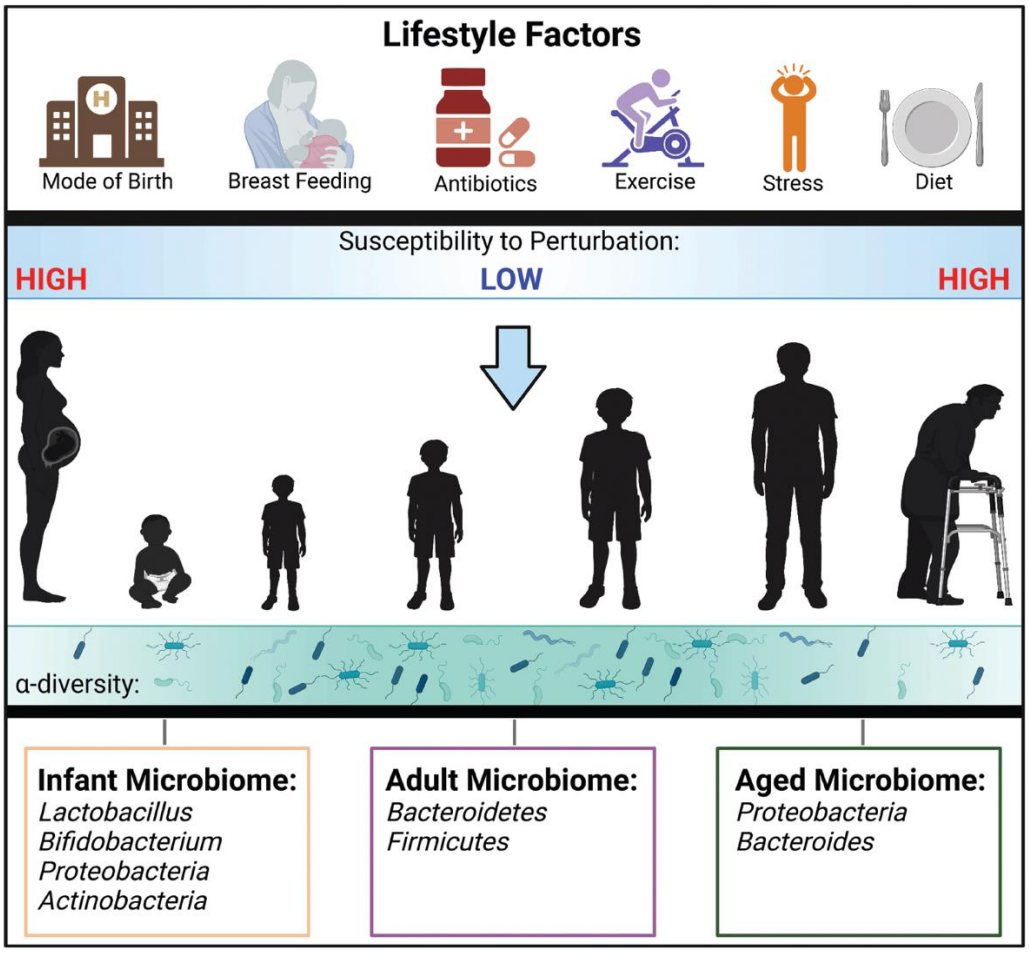

Figure 1.1. The gut microbiome across the lifespan. The composition of the gut microbiome evolves throughout the lifespan and is shaped by many lifestyle and environmental factors.

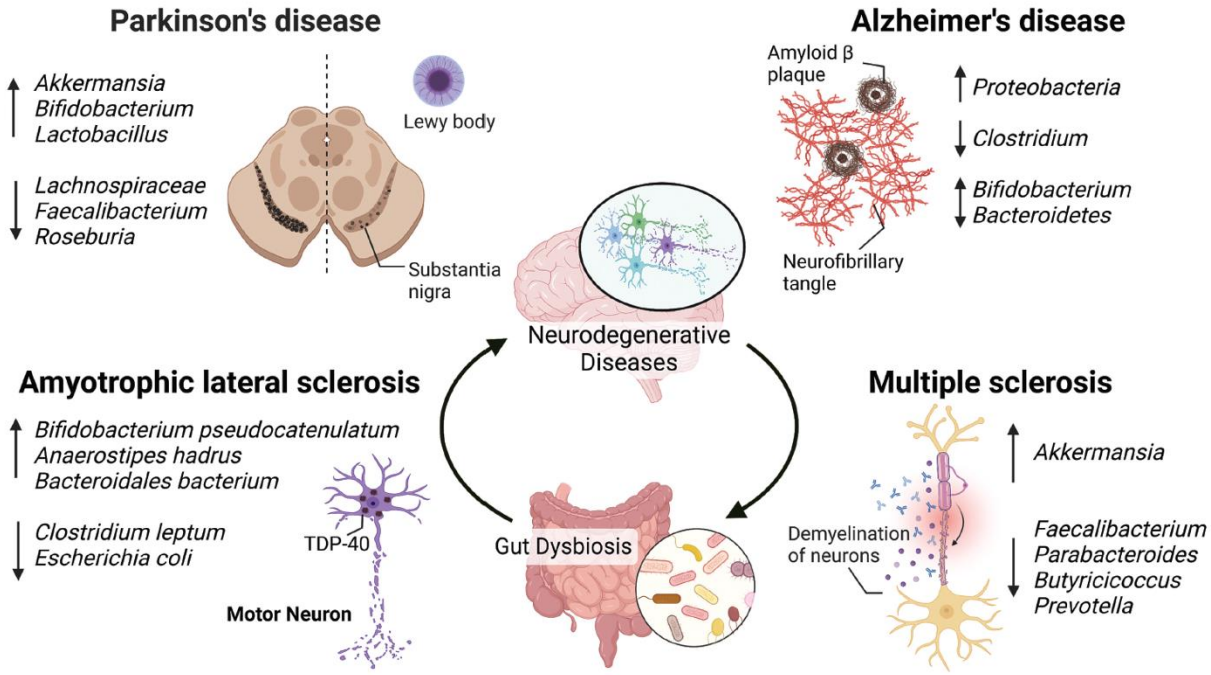

Figure 1.2. The gut microbiome and neurodegenerative disease. Changes in gut microbiome composition are associated with several neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and multiple sclerosis.

Chapter 2: Alzheimer's Disease and the Microbiome

2.1 Introduction to Alzheimer's disease

Almost seven million people in the United States are currently living with Alzheimer's disease (AD), a number that is projected to rise to nearly 13 million by 2050(Alzheimer's Association 2023). AD is the number one cause of dementia and the fifth leading cause of death among people 65 and older(Alzheimer's Association 2023). The prevalence of AD is heavily influenced by race, with Black Americans having 2x greater risk of developing AD and Hispanic Americans having a 1.5x greater risk compared to White Americans. Further, almost two-thirds of AD patients are women(Alzheimer's Association 2023). This incredibly prevalent and debilitating dementia results in significant reductions in quality of life for both patients and caregivers and an annual economic burden of $360 billion in the United States alone(Alzheimer's Association 2023).

2.2 Alzheimer's disease symptoms and pathology

AD is a neurodegenerative disorder in which patients experience progressively worsening cognitive impairment. Early symptoms of AD include difficulty remembering names or recent events as well as affective changes such as depression or anxiety(Alzheimer's Association 2023). As the disease progresses, memory problems continue to worsen, and patients experience personality changes, confusion, and executive dysfunction(Alzheimer's Association 2023). Finally, in late stages of the disease, patients develop difficulty walking, speaking, and swallowing(Alzheimer's Association 2023). Symptomatic illness typically lasts 8-10 years, but pathology is thought to begin developing decades prior to symptom onset(Masters, Bateman et al. 2015). The pathological hallmarks of AD consist of amyloid beta (Aβ) plaques that accumulate extracellularly and neurofibrillary tangles consisting of hyperphosphorylated tau within neurons. Amyloid pathology is thought to develop before tauopathy, primarily in the frontal and temporal lobes, hippocampus, and limbic system(Braak and Braak 1997). Neurofibrillary tangles are thought to develop later, starting in the medial temporal

lobe and hippocampus and then progressing to adjoining regions of the neocortex(Braak and Braak 1997). Neuroinflammation is also a prominent feature of AD pathology. Microglia—the primary immune cells of the brain—are though to play a dual role in AD development, first aiding in the clearance of Aβ plaques and providing neurotrophic support, but as the disease progresses, chronic inflammation leads to neuronal damage and oxidative stress, Aβ accumulation, synapse loss, and blood brain barrier breakdown(Leng and Edison 2021).

2.3 Alzheimer's disease etiology

The causes of AD remain poorly understood, but are thought to be due to a combination of genetic and environmental factors. A small subset of AD patients (less than 1%) have rare, heritable forms of AD caused by mutations in genes such as APP, PSEN1, and PSEN2 which cause increased accumulation of Aβ and early onset AD(Masters, Bateman et al. 2015). The majority of AD patients, however, have sporadic, late onset AD (LOAD) without a clear singular cause. Although LOAD is not thought to be caused by a single genetic mutation, studies have identified numerous genes that increase the risk of developing AD. For example, the APOE4 allele is the greatest known genetic risk factor for AD and increases one's risk of developing AD by three to four times(Jansen, Savage et al. 2019). Other AD risk genes are primarily involved in cholesterol metabolism, endocytosis, and immune responses, highlighting the importance of these processes in AD(Karch and Goate 2015). In addition to genetic factors, several environmental risk factors are associated with AD. The 2020 Lancet Commission on dementia prevention, intervention, and care identified twelve modifiable environmental risk factors for dementia including lower education, hypertension, hearing impairment, smoking, obesity, depression, physical inactivity, diabetes, low social contact, excessive alcohol consumption, traumatic brain injury, and air pollution(Livingston, Huntley et al. 2020). Together, these factors are thought to account for around 40% of dementia cases worldwide(Livingston, Huntley et

al. 2020).

2.4 Alzheimer's disease treatments

Despite a century of research and over 2,000 clinical trials, AD has limited treatment options and no cure(Liu, Xie et al. 2019). Traditional AD treatments primarily involve the use of cholinesterase inhibitors to increase cholinergic signaling, compensating for loss of cholinergic neurons, and improving cognitive function(Liu, Xie et al. 2019). While initially effective, these medications do nothing to slow disease progression or treat the underlying cause(Liu, Xie et al. 2019). Recently, however, the FDA approved two novel potential disease modifying medications that directly target Aβ pathology. The first of these, aducanumab, was shown to dramatically reduce amyloid pathology in the brains of AD patients, but there was conflicting data as to whether this resulted in significant symptomatic improvement(Sevigny, Chiao et al. 2016, Knopman, Jones et al. 2021). The development and marketing of aducanumab has since been halted in favor of the newer, more promising anti-Aβ therapeutic lecanemab(Cox 2024). Unlike aducanumab, lecanemab was shown to both reduce amyloid pathology and slow cognitive decline in patients with early AD(Van Dyck, Swanson et al. 2023). However, lecanemab is also associated with an increased risk of amyloid-related imaging abnormalities which could potentially be harmful to some patients(Van Dyck, Swanson et al. 2023). While promising, lecanemab is still not a cure, and additional research to identify medications that halt, or reverse disease progression is sorely needed.

2.5 The microbiome in Alzheimer's disease

Although numerous smaller studies have been published attempting to characterize the AD microbiome, few metanalysis or large-scale studies have been performed. As such, there is a significantly less clear picture of what the AD-associated microbiome consists of, or if there is a

specific composition associated with AD. Despite these challenges, the few published studies identify some apparent compositional differences in those with AD. AD patients show a reduction in overall phylogenic diversity and a microbiome composition that is distinct from healthy controls(Vogt, Kerby et al. 2017, Liu, Wu et al. 2019, Ling, Zhu et al. 2020, Hung, Chang et al. 2022). Like in PD, several studies have demonstrated that AD patients have an increase in *Bifidobacterium* and the phylum *Actinobacteria* in which it resides(Zhuang, Shen et al. 2018, Ling, Zhu et al. 2020, Hung, Chang et al. 2022). In fact, *Bifidobacterium* was found to be positively correlated with worsened symptom severity(Ling, Zhu et al. 2020). Of note however, another prominent study found a significant decrease in *Bifidobacterium* in AD patients that was correlated with worsened amyloid and tau CSF biomarkers(Vogt, Kerby et al. 2017), an example of significant discrepancies remaining in this field. Another trend that has appeared in several studies is an increase in *Escherichia/Shigella* and their phylum *Proteobacteria*(Cattaneo, Cattane et al. 2017, Liu, Wu et al. 2019). Levels of *Escherichia/Shigella* were found to be positively correlated with serum pro-inflammatory cytokines in AD patients, suggesting a possible contribution of these bacteria to pathologic neuroinflammation(Cattaneo, Cattane et al. 2017). The family *Bacteroides* and phylum *Bacteroidetes* also appear different in AD, although the direction of change varies by geographical location(Vogt, Kerby et al. 2017, Zhuang, Shen et al. 2018, Hung, Chang et al. 2022). Finally, decreases in Clostridium have also been reported in several studies(Vogt, Kerby et al. 2017, Hung, Chang et al. 2022).

Increasing evidence from mouse models suggests that AD outcomes may be modulated by the gut microbiome. AD mouse models treated with high dose antibiotics(Minter, Zhang et al. 2016, Minter, Hinterleitner et al. 2017, Dodiya, Kuntz et al. 2019, Dodiya, Frith et al. 2020, Mezö, Dokalis et al. 2020, Dodiya, Lutz et al. 2022, Seo, O'Donnell et al. 2023) or raised in a germ-free environment(Harach, Marungruang et al. 2017, Mezö, Dokalis et al. 2020, Seo, O'Donnell et al.

2023) display significant reductions in amyloid and tau pathology, neurodegeneration, and neuroinflammation, highlighting the contribution of microbes to AD outcomes. Furthermore, the overall microbial composition that occurs in AD mouse models may play a role in the development of pathology. For example, colonization of antibiotic-treated or germ-free APP/PSEN1 mice with the microbiota of dysbiotic APP/PSEN1 mice exacerbates AD-like pathology(Harach, Marungruang et al. 2017, Dodiya, Kuntz et al. 2019) while colonization with healthy wild-type microbiota induces considerably less amyloid pathology(Harach, Marungruang et al. 2017). In addition, transfer of healthy wild-type microbiota to dysbiotic AD mouse models improves disease outcomes(Sun, Xu et al. 2019, Kim, Kim et al. 2020) while transfer of dysbiotic AD microbiota to wild-type mice induces cognitive impairment and neuroinflammation(Kim, Jeon et al. 2021). Although fecal microbiome transplantation (FMT) is not commonly used in AD patients, significant and long-lasting improvements in cognition following FMT to treat a *Clostridioides difficile* infection have been reported in several case studies (Hazan 2020, Park, Lee et al. 2021).

2.6 Alzheimer's disease, neuroinflammation, and the microbiome

Although the ways in which the microbiome shapes AD remains largely unexplored, there is growing evidence to suggest that neuroinflammatory signaling plays an important role. Depletion of myeloid cells from the brain—primarily microglia—eliminates the protective effects of antibiotic treatment on AD-associated outcomes (Dodiya, Lutz et al. 2022), suggesting that this cell type may serve as the conduit for microbiome communication in AD. Indeed, there is growing evidence that a) microglia play an essential role in AD and b) the microbiome modulates microglia activation and response to disease (Schaible, Henschel et al. 2025). Research on microglia in AD has demonstrated both beneficial and detrimental roles. Initially, microglia activation is thought to be protective, clearing toxic debris and maintaining homeostasis(Leng and Edison 2021). However, microglia can switch to

a pro-inflammatory state that causes prolonged release of neurotoxic molecules, stimulates excessive synapse pruning, and may in fact increase Aß aggregation and spread tau pathology (Leng and Edison 2021). Therefore, factors that predispose microglia to enter either a protective or neurotoxic state will significantly impact AD outcomes. Growing evidence suggests that microbiome composition may be one of these factors. Several studies have demonstrated that microglia from both GF and antibiotic treated mice display an immature phenotype and decreased innate immune response (Erny, Hrabe de Angelis et al. 2015, Matcovitch-Natan, Winter et al. 2016, Sampson, Debelius et al. 2016, Thion, Low et al. 2018, Mossad and Erny 2020, Mossad, Batut et al. 2022), highlighting how microbial signals continually shape microglia state. Furthermore, GF and antibiotic treated AD mouse models display altered microglia response to Aβ pathology, with changes in the morphology, gene expression, and Aβ uptake of plaque-associated microglia (Minter, Zhang et al. 2016, Minter, Hinterleitner et al. 2017, Dodiya, Kuntz et al. 2019, Mezö, Dokalis et al. 2020, Dodiya, Lutz et al. 2022).

In addition to changes in microglia, increasing evidence suggests that non-microglia immune cells may also play a critical role in AD outcomes. Systemic infection and chronic inflammation is linked to increased AD susceptibility and worsened outcomes (Xie, Van Hoecke et al. 2022). The peripheral immune system is dependent on the gut microbiome, with GF mice having increased susceptibility to infectious disease (Round and Mazmanian 2009). These impacts are particularly significant within the adaptive immune system, that is said to be trained to properly respond to immune stimuli by the microbiome. Indeed, in the absence of indigenous microbes, GF mice have smaller, poorly developed lymphoid organs, limited numbers of lymphocytes, impaired lymphocyte maturation, and a significant reduction in IgA antibody production (Round and Mazmanian 2009, Gensollen, Iyer et al. 2016). Highlighting the species specific consequences of bacteria on the peripheral immune system, Geva-Zatorsky et al., have shown that specific bacteria have very distinct, effects on the peripheral immune system, even into adulthood (Geva-Zatorsky, Sefik et al. 2017).

While the role of the adaptive immune system in AD didn't begin to be fully recognized until recently, increasing evidence suggests that the adaptive immune system plays an important role in modulating AD outcomes (Chen and Holtzman 2022). Increased circulating levels of $CD8^+$ T effector memory $CD45RA^+$ (T_{EMRA}) cells were found in AD patient blood samples and were negatively associated with cognition (Gate, Saligrama et al. 2020). $CD8^+$ T cells were also found to be elevated in the cerebrospinal fluid (Gate, Saligrama et al. 2020) and brains of AD patients (Costa 2024, Yamakawa and Rexach 2024). In both amyloid and tau-based AD mouse models, increased T cell infiltration into the brain is associated with worsened AD outcomes (Laurent, Dorothée et al. 2016, Chen, Firulyova et al. 2023, Kedia, Ji et al. 2024, Panwar, Rentsendorj et al. 2024, Wang, Campbell et al. 2024, Zeng, Liao et al. 2024). T cell infiltration is thought to be triggered by myeloid cells in the brain, and myeloid cell depletion has been shown to reduce T cell infiltration, neurodegeneration, and tau pathology in AD mice (Chen, Firulyova et al. 2023). The activation and recruitment of T cells is driven by antigens presented on MHC class I and II proteins, which classically present to $CD8^+$ and $CD4^+$ T cells respectively (Neefjes, Jongsma et al. 2011). In the context of neurodegeneration however, increased MHCII antigen presentation by microglia or border associated macrophages (a brain resident macrophage population that resides along the borders of the central nervous system (CNS), serving as the initial point of communication between the central and peripheral immune system) is associated with both increased $CD4^+$ and $CD8^+$ T cell infiltration and exacerbated disease outcomes (Williams, Schonhoff et al. 2021, Chen, Firulyova et al. 2023, Schonhoff, Figge et al. 2023). Although the consequences of the microbiome for adaptive immune responses within the brain remain poorly understood, it is likely that the effects of the microbiome on both peripheral adaptive immune processes and myeloid cell activation state would translate to adaptive immune changes within the CNS as well.

2.7 Dissertation aims

Increasing evidence highlights capacity of the gut microbiome to shape neurological function in both health and disease, however, how the microbiome simultaneously shapes the transcriptional landscape across different brain-resident cell populations remains poorly understood. Furthermore, despite the various associations of specific bacterial taxa with neurological diseases, few studies have addressed physiological contributions of individual microbial species to relevant neuroimmune and neurodegeneration related outcomes. The present dissertation aims to address these gaps through three specific aims: 1) I performed the first single-cell characterization of microbiome-dependent transcriptional state across all major cell types within the mouse brain, helping to identify both the conserved and cell-type specific transcriptional changes modulated by microbiome-derived signals; 2) I characterized the neuroimmune modulatory capacity of four representative gut-resident bacteria, identifying bacteria specific neuroimmune responses; 3) As proof of concept, I performed an in depth characterization of the transcriptional landscape of the brain after *Escherichia coli* colonization and demonstrated that *E. coli* is sufficient to modify disease outcomes in a mouse model of AD. Together these results highlight the widespread impact of native microbiome-dependent signaling on the brain, as well as the specific consequences of individual gut microbes for neuroimmune and neurological function, emphasizing the importance of the native microbiome in shaping transcriptional tone and disease susceptibility.

Chapter 3: Indigenous gut microbes modulate neural cell state and neurodegenerative disease susceptibility

This chapter is reproduced with minor edits from: **Blackmer-Raynolds L**, Sampson MM, Kozlov A, Yang A, Lipson L, Hamilton AM, Kelly SD, Chopra P, Chang J, Sloan SA, Sampson TR. Indigenous gut microbes modulate neural cell state and neurodegenerative disease susceptibility. bioRxiv. 2025 Feb 22:2025.02.17.638718. doi: 10.1101/2025.02.17.638718. PMID: 40027785. The author designed the and ran experiments, analyzed the data, and wrote the manuscript. Sampson MM, P Chopra, and Sloan SA provided guidance for RNA-seq library preparation and data analysis. Kozlov A and Lipson L performed immunohistochemistry and cytokine analysis. Yang A assisted with behavioral testing. AM Hamilton helped with animal colonization/care and gastrointestinal assessments. Kelly SD helped with immunohistochemistry. Chang J assisted with animal husbandry and multiplex ELISA. Sampson TR helped procure funding, design and supervise experiments, and write the paper.

3.1. Abstract

The native microbiome influences a plethora of host processes, including neurological function. However, its impacts on diverse brain cell types remains poorly understood. Here, we performed single nucleus RNA sequencing on hippocampi from wildtype, germ-free mice and reveal the microbiome-dependent transcriptional landscape across all major neural cell types. We found conserved impacts on key adaptive immune and neurodegenerative transcriptional pathways, underscoring the microbiome's contributions to disease-relevant processes. Mono-colonization with select indigenous microbes identified species-specific effects on the transcriptional state of brain myeloid cells. Colonization by *Escherichia coli* induced a distinct adaptive immune and neurogenerative disease-associated cell state, suggesting increased disease susceptibility. Indeed, *E. coli* exposure in the 5xFAD mouse model resulted in exacerbated cognitive decline and amyloid pathology, demonstrating its sufficiency to worsen Alzheimer's disease-relevant outcomes. Together, these results emphasize the broad, species-specific, microbiome-dependent consequences on neurological transcriptional state and highlight the capacity of specific microbes to modulate disease susceptibility.

3.2. Introduction

The human body is colonized by a diverse and complex community of microbes, the microbiome, that shapes a range of host physiological processes. An individual's gastrointestinal (GI) tract harbors 100-500 unique bacterial species that are influenced by genetic, environmental, and lifestyle factors, with an estimated 3,500 unique strains of human gut-resident bacterial species worldwide(Qin, Li et al. 2010, Scepanovic, Hodel et al. 2019, Leviatan, Shoer et al. 2022, Blackmer-Raynolds and Sampson 2023). Given this variability in microbiome composition between individuals, across the lifespan, and within the context of disease, understanding the physiological

consequences of specific microbial taxa on health outcomes, particularly neurological disease, is essential.

Gnotobiotic mouse models raised in a sterile, germ-free (GF) environment, or treated with high dose antibiotics have provided significant insights into the contributions of the gut microbiome to host physiology. For instance, in the absence of indigenous microbes, GF mice have smaller, poorly developed lymphoid organs; limited lymphocyte maturation; and increased susceptibility to infectious disease(Round and Mazmanian 2009, Gensollen, Iyer et al. 2016). In the brain, GF or antibiotic-treated mice harbor immature microglia that are less capable of mounting inflammatory responses and defending against pathogens(Erny, Hrabe de Angelis et al. 2015, Matcovitch-Natan, Winter et al. 2016, Thion, Low et al. 2018). In addition to—or perhaps even as a result of—microbiome contributions to neuroinflammatory tone, the microbiome also has broad impacts on neurological function in the context of both health and disease. A native microbiome is necessary for proper neurogenesis, myelination, and neurotransmitter production; with impacts on anxiety-like, social, and cognitive behaviors in mice(Cryan, O'Riordan et al. 2019). While some microbiome-dependent effects on immune and neurological functions are developmental and irreversible, others are quickly restored by microbial colonization or exposure to microbial metabolites, highlighting the importance of continuous microbial input for proper neurological functions into adulthood (Erny, Hrabe de Angelis et al. 2015, Gensollen, Iyer et al. 2016, Sharon, Sampson et al. 2016).

Whole microbiome manipulations in mouse models of disease have significant impacts on both behavioral and pathological outcomes(Cryan, O'Riordan et al. 2020, Fang, Kazmi et al. 2020). In the absence of an intact, native microbiome, mouse models of Alzheimer's disease (AD) show significant improvements in cognitive performance and reductions in both amyloid beta (Aß) and tau pathology(Minter, Zhang et al. 2016, Harach, Marungruang et al. 2017, Minter, Hinterleitner et al. 2017, Dodiya, Kuntz et al. 2019, Dodiya, Frith et al. 2020, Mezo, Dokalis et al. 2020, Dodiya,

Lutz et al. 2022, Seo, O'Donnell et al. 2023). Depletion of myeloid cells from the brains of antibiotic-treated APPPS1-21 mice eliminates the protective effect of antibiotic treatment, suggesting that microbiome-mediated neuroimmune signaling contributes to pathological outcomes(Dodiya, Lutz et al. 2022). While these studies emphasize the microbiome's capacity to modulate overall brain health and function, how the microbiome shapes the transcriptional state across different brain-resident cell populations, remains poorly understood. Furthermore, despite the various associations of specific bacterial taxa with neurological diseases(Fang, Kazmi et al. 2020), few studies have addressed physiological contributions of individual microbial species to relevant neuroimmune functions.

Here, we provide a single-cell characterization of microbiome-dependent transcriptional state within the mouse brain and identify both the conserved and cell-type specific transcriptional landscapes temporally modulated by microbiome-derived signals. In particular, the neuroimmune compartment was one of the most transcriptionally responsive, suggesting that these cells act to propagate signals from the microbiome to other cells within the CNS. These responses are specific, as we find that select, non-pathogenic, gut-resident species are sufficient to uniquely shape the transcriptional state of brain-resident myeloid cells. In particular, the species *Escherichia coli* induces a distinct, and temporally modulated transcriptional profile across not only brain-resident immune cells, but also across neuronal and other glial populations. Colonization with *E. coli* dynamically regulates neurodegenerative disease associated pathways across numerous neural cell types. In fact, oral exposure to *E. coli* worsens cognitive impairments and increases amyloid pathology within the 5xFAD mouse model of AD, underscoring the pathological relevance of the microbiome-dependent transcriptional landscape. Together these results highlight the widespread impact of native microbiome-dependent signaling on the brain, as well as the specific consequences of individual gut microbes for neurological function, emphasizing the importance of the native microbiome in

shaping transcriptional tone that can impact disease.

3.3. Results

3.3.1. A complex microbiome is necessary for the steady-state transcriptional landscape across many brain-resident cell types.

In order to understand how select gut bacterial species impact the brain, we first sought to determine how the complete absence of an indigenous microbiome influenced the cellular transcriptional landscape. We performed single-nucleus RNA sequencing (snRNAseq) on hippocampal tissues derived from young adult female, germ-free (GF) or conventional (CONV) mice with a complete and intact microbiome. Unsupervised clustering of 23,963 total nuclei (representing 4 mice per treatment group) revealed seven unique clusters, roughly equally represented across each microbiome status (Fig. 3.1A), that were identified as the following cell types based on marker genes(Alzheimer's Association): excitatory neurons (Cluster 1), inhibitory neurons (Cluster 2), astrocytes (Cluster 3), myelinating oligodendrocytes (Cluster 4), vasculature (Cluster 5), oligodendrocyte progenitor cells (OPCs; Cluster 6), and immune cells (Cluster 7) (Fig. 3.1B; Supplementary Data S1 - available online at DOI: 10.1101/2025.02.17.638718). Differential gene expression analysis demonstrated microbiome dependent transcriptional responses within each cluster, with most differentially expressed genes (DEGs) occurring within excitatory neurons, myelinating oligodendrocytes, vasculature cells, and immune cells (Fig 3.1C; Supplementary Data S1 - available online at DOI: 10.1101/2025.02.17.638718). Upon normalization by cluster size, to further account for the increased power in highly abundant clusters, myelinating oligodendrocytes and immune cells were found to have the largest microbiome-dependent transcriptional response (Fig 3.1D. This highlights these cell types' unique susceptibility at the interface between the CNS and periphery to indigenous microbial signals.

To evaluate the biological relevance of these microbiome-dependent DEGs, we performed

pathway analysis (Fig. 3.1E, F; Supplementary Fig. S3.1; Supplementary Data S1 - available online at DOI: 10.1101/2025.02.17.638718). We first examined biological pathways conserved in their microbiome-dependent responses across at least 4 cell types. While we did not observe any pathway decreased within 4 cell types, we did find a number of shared, microbiome-dependent features within those biological pathways enriched in many cell types (Fig. 3.1F). Among all cell clusters, mRNA processing (GO:0006397) was highly enriched in the absence of a microbiome. Across 4 to 5 cell types, we observed a further enrichment in protein localization (GO:0010954) and membrane trafficking (R-MMU-199991) pathways, adaptive immune system (R-MMU-1280218), cell cycle (R-MMU-1640170) and brain development (GO:0007420) pathways, indicating a widespread repression of these particular signaling pathways by the native microbiome. These observations align with prior, targeted studies which demonstrated microbiome-dependent impacts on brain development and organization(Cryan, O'Riordan et al. 2019) and neuroimmune activation(Erny, Hrabe de Angelis et al. 2015, Matcovitch-Natan, Winter et al. 2016, Thion, Low et al. 2018). Even within the pathways that were increased in less than 4 cell types, impacted pathways were conceptually related, falling broadly within the categories of development, cellular stress, and immune processes (Supplementary Fig. S3.1A). Within both excitatory and inhibitory neuron clusters, gene set enrichment analysis (GSEA) of KEGG pathways revealed a significant microbiome-dependent enrichment of all major neurodegeneration-associated KEGG pathways (Fig. 3.1E). These same cell types also displayed increased immune related pathways (*e.g.* adaptive immune system (R-MMU-1280218), neutrophil degranulation (R-MMU-6798695), cellular response to interlukin-4 (GO:0070670), and interferon signaling (R-MMU-913531), among others), emphasizing the interaction between the indigenous microbiome, neuroimmune activation, and neurodegenerative disease pathways in shaping the neuronal transcriptional landscape (Supplementary Fig. S3.1A).

3.3.2. Select gut microbes differentially and specifically modulate the brain-resident myeloid cell transcriptome.

Immune cells within the brain are observed to be particularly susceptible to perturbations within the gut microbiome(Erny, Hrabe de Angelis et al. 2015, Matcovitch-Natan, Winter et al. 2016, Thion, Low et al. 2018) (Fig. 3.1D). The increases we found in immune related pathways across brain cell types, suggest that immune cells are critical for transducing microbiome-derived signaling to other cells within the brain. However, it is unclear whether these activities are a generalized response to broad bacterial organisms or if specific, indigenous microbes are sufficient to impart differential effects on brain-resident immune cells. Therefore, we set out to test the specificity and sufficiency of select, indigenous gut bacterial species to modulate the transcriptional state of brain-resident myeloid cells (primarily microglia). Wild-type GF mice were mono-colonized with select bacterial type strains representing prevalent genera within the mammalian gut microbiome-*Bacteroides thetaiotaomicron* (*B. theta*)*, Clostridium celatum, Lactobacillus johnsonii,* and *Escherichia coli*- for 2 weeks. CD11b+ myeloid cells, largely representing microglia but also present on other leukocytes, were then enriched(Van Hove, Martens et al. 2019), and bulk transcriptomics was performed (Fig. 3.2A).

Differential gene expression analysis of CD11b+ brain myeloid cells demonstrated that all but one of the bacteria studied—*L. johnsonii*—were sufficient to modulate myeloid cell gene expression, with colonization by *E. coli* and *B. theta* inducing the most DEGs (Fig. 3.2B; Supplementary Data S2 - available online at DOI: 10.1101/2025.02.17.638718). Comparison of the DEGs increased and decreased after mono-colonization shows very little overlapping DEGs, highlighting the specificity of the transcriptional response to each of these unique bacterial species (Fig. 3.2C). No shared DEGs were increased by all three species and of the decreased genes, only 10 were shared across colonization states (Fig. 3.2C). Further emphasizing the unique transcriptional state induced by each bacterial species, pathway analysis comparing across each bacterial

colonization state shows near inverse effects between the unique species (Fig. 3.2D; Supplementary Data S2 - available online at DOI: 10.1101/2025.02.17.638718). Where *E. coli* induced an increase in pathways involved in immune activation and adaptive immune responses, *B. theta* and *C. celatum* decreased many of these same pathways in male mice. Further emphasizing the unique transcriptional response of brain myeloid cells to *E. coli*, comparison of KEGG pathway enrichment revealed that *E. coli* mono-colonization triggered an inverse effect to that of *B. theta* or *C. celatum* amongst all shared KEGG pathways (Fig. 3.2E). To validate the transcriptional increase in genes involved in adaptive immune processes, specifically MHCII antigen presentation, immunohistochemistry was performed to evaluate MHCII levels within the brain of male mono-colonized mice (Fig. 3.2F). MHCII+ cells were primarily found within CD206+ cells, canonically considered to be border associated macrophages, within the CD31+ choroid plexus of the lateral ventricle. In line with the transcriptional data, when compared to GF mice, *E. coli* mono-colonization induced significantly more MHCII+ / CD206+ double-positive cells (Fig. 3.2F), supporting the increase in adaptive immune transcriptional processes within the brains of these animals. In addition to pathways involved in adaptive immune responses, *E. coli* alone induced an increase in KEGG pathways involved in neurodegenerative diseases—including prion disease (mmu05020), AD (mmu05010), and Amyotrophic lateral sclerosis (ALS, map05014)—highlighting the potential for *E. coli* colonization to modulate a disease-susceptible transcriptional state within the brain (Fig. 3.2G). Notably, multiplex measurement of cytokines in both intestinal tissues and serum demonstrated limited impacts to inflammatory cytokine levels (Supplementary Figure S3.2A, B). While a decrease in ileal TNF and increase in colonic CXCL1 and TNF was observed in most mono-colonized conditions (Supplementary Fig. S3.2A), we observed no overt effect in colon length or other measure of GI function (Supplementary Fig. S3.2C-E). In conjunction with no observed effect on circulating cytokines during colonization by any tested microbe (Supplementary Fig.

S3.2B), these data suggest that mono-colonization did not induce robust local or systemic inflammatory signaling. Similarly, no overt gliosis was observed, with limited impact on the IBA1+ area (Supplementary Fig. S3.2F, G), demonstrating transcriptional effects in the absence of robust gliosis or systemic inflammation.

3.3.3. *E. coli* elicits a temporal transcriptional response across CNS-resident cells

Given the experimental contributions of *E. coli* to pathologies in models of neurodegenerative disease(Chen, Stribinskis et al. 2016, Sampson, Challis et al. 2020, Chongtham, Yoo et al. 2022, Liang, Liu et al. 2023), and our observations here that this native microbe is sufficient to drive both adaptive immune and neurodegenerative disease-associated pathways in brain-resident myeloid cells, we next sought to understand how this organism dynamically shapes transcriptional tone across many neural cell types. We performed snRNA-seq on hippocampal samples from female mice mono-colonized with *E. coli* for either 2 or 4 weeks to capture both short- and longer-term transcriptional responses following microbial colonization in comparison to GF controls. Clustering of 30,093 total nuclei (representing 4 mice per treatment group) was performed in combination with previously assessed nuclei (Fig. 3.1) to create comparable cell clusters (Fig. 3.3A, B; Supplementary Data S3 - available online at DOI: 10.1101/2025.02.17.638718). Interestingly, while cell numbers between each cluster appeared relatively unchanged following 2 weeks of *E. coli* colonization, by 4 weeks we observed a qualitative shift in cell populations. This is highlighted by over an 18.5% decrease in excitatory neuron representation and a corresponding increased representation of inhibitory neurons, myelinating oligodendrocytes, and astrocytes (Fig. 3.3A).

Differential gene expression analysis showed time dependent shifts in the number of DEGs increased in each cell type compared to GF (Fig. 3.3C; Supplementary Data S3 - available online at

DOI: 10.1101/2025.02.17.638718). Where the immune cell cluster displayed the most increased DEGs and myelinating oligodendrocytes the most decreased DEGs at 2 weeks post-colonization, the number of DEGs in both of these cell types was dramatically reduced at 4 weeks. In contrast, the number of DEGs in the other cell clusters (excluding OPCs that remained largely unresponsive) was dramatically higher at 4 weeks compared to 2 weeks. While many of the DEGs that were significantly altered at 2 weeks in the immune cell and myelinating oligodendrocyte clusters began to stabilize back towards a non-colonized state at 4 weeks (Fig. 3.3D), they did not fully recover, highlighting long-term, yet subtle, transcriptional states that may alter susceptibility to future insults. In contrast, the neuron clusters—with the highest number of DEGs at 4 weeks (Fig. 3.3C)—displayed a limited transcriptional response until 4-weeks post-colonization with *E. coli* (Fig. 3.3D). We interpret this to suggest that these cells are either slower to respond to microbiome status or instead, respond to those signals derived from more acutely responsive cells.

Since we observed the immune cell cluster as having the most increased DEGs in the early colonization state at 2 weeks post-*E. coli* colonization (Fig. 3.3), and bulk RNA-seq of brain myeloid cells displayed a microbiome-dependent adaptive immune and neurodegenerative disease phenotype (Fig. 3.2), we hypothesized that this cell type may initiate the broader transcriptional responses in other cells throughout the brain. In order to distinguish the microbiome dependent effects on microglia compared to other immune cell types, we performed sub-clustering and pathway analysis within the immune cell cluster (Fig. 3.4; Supplementary Data S4 - available online at DOI: 10.1101/2025.02.17.638718). Unsupervised sub-clustering identified 2 main sub-clusters: microglia and non-microglia immune cells (*e.g.* border associated macrophages, infiltrating peripheral immune cells) (Figs. 3.4A-C). While similarly prevalent irrespective of colonization status, non-microglia immune cells were lowly abundant and showed little transcriptional responsiveness to *E. coli* across both time points (Fig. 3.4D), however, this may be due to an insufficient sample size , rather than a

lack of susceptibility to perturbation.

In contrast, differential gene expression analysis identified 515 repressed and 78 induced genes in microglia following 2-weeks of mono-colonization (Fig. 3.4E). Pathway analysis on these DEGs identified similar pathways as we observed in our analysis of CD11b+ cells (Fig. 3.2) including an increase in pathways involved in inflammatory responses and adaptive immunity, whereas downregulated pathways included those characterized as cellular organization and mRNA processing (Fig. 3.4F). At 4-weeks post-colonization, differential gene expression analysis of microglia revealed 23 increased and 142 decreased DEGs (Fig. 3.4G). We observed a repression in genes involved mRNA metabolic processes and cellular organization, as we observe at the 2-week time point. However, microglia also showed a decrease in cellular stress pathways (*e.g.* regulation of double stranded break repair (GO:2000779) and stress granule assembly (GO:0034063)) at 4-weeks post-colonization (Fig. 3.4H). Further, GSEA of microglia at the 4-week timepoint displayed a significant decrease in a range of neurodegenerative disease pathways (Fig. 3.4I), contrasting with the *E. coli* triggered increase in these pathways we observed at 2-weeks (Fig. 3.2G).

To better understand the breadth of the transcriptional response to *E. coli* colonization, we performed pathway analysis within every other cell cluster at each timepoint (Fig. 3.5A, B; Supplementary Data S5 - available online at DOI: 10.1101/2025.02.17.638718). At both 2- and 4-weeks post-colonization, we observed a significant decrease in pathways involved in RHO GTPase signaling, adaptive immunity, and RNA metabolism across cell types. This mirrors our observations in mice with a complex, and intact microbiome (Fig. 3.1E), and highlights that these transcriptional pathways may be highly sensitive to microbial colonization. In contrast, pathways involved in nervous system / brain development were increased in *E. coli* colonized mice, while these were decreased in conventional mice compared to GF animals (Fig. 3.1E). Cell-specific pathways further highlight that colonization with *E. coli* induced a time-dependent response in immune, cellular stress,

and cellular organization/transport pathways (Supplementary Fig. S3.3).

Since these transcriptional responses are relevant for neurological disease, we specifically examined neurodegenerative disease KEGG pathways by both overrepresentation pathway analysis and GSEA (Fig. 3.5C). We observed a modulation of neurodegeneration KEGG pathways across nearly every cell type following *E. coli* colonization, with a decrease in KEGG pathways of neurodegenerative diseases in both excitatory and inhibitory neurons following colonization across both timepoints, and in microglia by 4-weeks post-colonization (Fig. 3.5C). In addition, overrepresentation-based pathway analysis demonstrated an enrichment of neurodegeneration pathways in both astrocytes and vasculature at 4 weeks of colonization (Fig. 3.5C). Taken together, these results emphasize a link between *E. coli* colonization and transcriptional pathways that are involved in modulation of neurodegenerative disease risk.

3.3.4. *E. coli* modulates cognitive impairment in an animal model of amyloid pathology

We have demonstrated that *E. coli* is sufficient to transcriptionally modulate adaptive immune and neurodegenerative disease pathways across many cell types in the brain. Notably, *E. coli* and closely related organisms have been reported to be enriched within the gut microbiome of individuals with neurodegenerative disease(Cattaneo, Cattane et al. 2017, Liu, Wu et al. 2019, Fang, Kazmi et al. 2020, Khedr, Omeran et al. 2022, Wallen, Demirkan et al. 2022, Wang, Li et al. 2022), suggesting it may contribute to disease processes. We therefore sought to directly test the disease modulatory potential of *E. coli* within the context of AD—the most prevalent neurodegenerative disease. To evaluate the sufficiency of gastrointestinal *E. coli* to modulate AD outcomes, we used the well-characterized 5xFAD mouse model(Oakley, Cole et al. 2006), that displays amyloid pathology beginning at 2 months of age(Richard, Kurdakova et al. 2015) and cognitive impairment between 3 and 6 months of age(Jawhar, Trawicka et al. 2012). To test exacerbation over this model's

pathological progression, 2-month-old conventionally raised 5xFAD mice were orally exposed to ~10^8 cfu of a non-pathogenic, strain of *E. coli* (identical to our mono-colonization experiments) or vehicle control 3x weekly for 1 month prior to behavioral and pathological assessments (Fig. 3.6A). Irrespective of treatment, 5xFAD mice displayed similar outcomes in both the open field and intestinal behaviors, suggesting that *E. coli* exposure did not induce overt sickness or anxiety-like behaviors, and GI functions were not robustly disrupted (Supplementary Fig. S3.4 A-E). While working memory, as measured in the Y-maze test, appeared intact (Fig. 3.6B), *E. coli* exposure resulted in a loss of novelty preference in the object location test (Fig 3.6C). Similarly, while learning capacity during Barnes Maze training was not impacted (Fig. 3.6D), *E. coli* treated 5xFAD mice displayed a significantly longer primary latency during the probe trial suggesting a loss of typical memory function (Fig. 3.6E). These observations appeared specific to the 5xFAD genotype, as wildtype littermates did not demonstrate any loss of cognitive functions in an identical battery of tests (Supplementary Fig. S3.5). Thus, in a genotype specific fashion, exposure to intestinal *E. coli* is sufficient to exacerbate cognitive decline in this mouse model.

To evaluate whether intestinal *E. coli* exacerbates pathological outcomes associated with the cognitive impairments observed in 5xFAD mice, we measured hippocampal Aß concentrations (Fig. 3.6 F-I). Where there were no differences in the concentration of Tris- or triton-soluble Aß (Fig. 3.6F-G), *E. coli* treated animals had significantly more formic acid soluble Aß (representing Aß in a highly insoluble form) than the vehicle treated controls (Fig. 3.6H), along with a trend towards increased Aß 42:40 ratio (Fig. 3.6I). Together, these data suggest that *E. coli* exposure exacerbates insoluble amyloid deposition. Profiling of cytokines and chemokines across anatomical compartments including the colon, serum, and hippocampus, demonstrated that exposure to *E. coli* did not induce a robust pro-inflammatory response in any anatomical compartment, in line with our behavioral evaluation for sickness-like behaviors (Supplementary Fig. S3.4 F-H).

Bulk RNA-seq of brain-derived CD11b+ cells from 5xFAD mice demonstrated a disease relevant transcriptional response to *E. coli* exposure. Brain myeloid cells, derived from *E. coli* treated mice had 12 repressed DEGs (Fig. 3.6J). Despite a small number of DEGs, nearly all are highly relevant for AD. For example, Apoe—the greatest known genetic risk factor for late onset AD(Serrano-Pozo, Das et al. 2021)—had one of the largest log fold change values, and all but two of the DEGs (Bhlhe40 and Rab7b) are known to be increased in the classical disease associated microglia (DAM) phenotype(Keren-Shaul, Spinrad et al. 2017). In support of this association, GSEA highlights a significant decrease in DAM genes following *E. coli* exposure (Fig. 3.6K). This suggests that *E. coli* exposure results in an inability for brain myeloid cells to transition into the initially protective DAM state and may explain the worsened cognitive behaviors and pathology observed in *E. coli* exposed 5xFAD mice. Overall, these results highlight how gut exposure to non-pathogenic *E. coli* alters brain wide transcriptional state in healthy wildtype animals and accelerates disease progression in a genetic model of AD.

3.4. Discussion

Increasing experimental evidence demonstrate that the gut microbiome maintains constant communication with the brain, shaping neurological function in both health and disease(Cryan, O'Riordan et al. 2019, Morais, Schreiber et al. 2021). We find that the gut microbiome shapes the transcriptional landscape of every major cell type in the brain, highlighting the breadth of microbiome-derived signaling. At a single cell resolution, we identify both cell-type specific and conserved transcriptional responses dependent on the presence of an intact microbiome. Further, our data delineates the shared and unique transcriptional responses elicited by particular gut microbial taxa, demonstrating the capacity for specific microbes to evoke distinct responses that are relevant for health and disease. Colonization with *E. coli* induces a broad transcriptional activation

state associated with adaptive immune and neurodegenerative disease pathways and further exacerbates disease outcomes in a mouse model of AD. Together the present study highlights the association between the gut microbiome community, the active transcriptional landscape in the brain, and neurological disease susceptibility.

Over the past several decades, numerous studies have underscored the importance of the gut microbiome in shaping neurological function including consequences for a wide range of neurological cell types(Cryan, O'Riordan et al. 2019, Morais, Schreiber et al. 2021). In line with this, we observe a consistent pattern, shared across cell types, of microbiome-dependent influences on developmental and cellular organization processes that are not typically found within adult mouse brains. Previous studies have demonstrated that microbiome-derived signals are necessary for appropriate neuroimmune development, with GF mice displaying an immature microglia phenotype and an inability to mount a typical inflammatory response(Erny, Hrabe de Angelis et al. 2015, Matcovitch-Natan, Winter et al. 2016, Thion, Low et al. 2018). Our data demonstrate a global dysregulation of genes associated with adaptive immune processes in the brain, in the absence of a microbiome, further emphasizing the importance of the microbiome for neuroimmune processes. Even in healthy, wildtype mice, both excitatory and inhibitory hippocampal neurons display dysregulated transcription of genes assigned to neurodegenerative disease pathways. These findings support the emerging role of the microbiome in modulating neurodegenerative disease outcomes, including numerous observations of microbiome-dependent pathology in both genetic and toxicant-induced models of neurodegenerative disease(Cryan, O'Riordan et al. 2020, Fang, Kazmi et al. 2020).

The composition of the gut microbiome differs across individuals, including significant differences in those living with neurological conditions(Blackmer-Raynolds and Sampson 2023). It is therefore important to not only understand the consequences of the microbiome as a whole, but also to pinpoint the specific effects of individual gut microbes on neurological functions. This allows

a deeper understanding of whether and how particular microbial associations may ultimately contribute to neurological outcomes. Indeed, we found species-specific effects on transcriptional activation of neuroimmune cells, including one organism *L. johnsonii*, whose colonization induced no transcriptional modulation compared to GF controls. This surprising finding demonstrates that neuroimmune cells do not simply respond broadly and non-specifically to microbial colonization, but that there is indeed specificity in these interactions arising from the gut environment. Three other organisms, *B. theta, C. celatum,* and *E. coli* each induced unique transcriptional responses, demonstrating the specific neuroimmune modulatory capacity of these GI resident bacterial taxa. *E. coli* notably triggered an increase in expression of genes involved in antigen presentation and adaptive immune activation, pathways which were strikingly decreased with colonization by both *B. theta* and *C. celatum*. Similarly, colonization with *E. coli* induced expression of several pathways associated broadly with neurodegenerative diseases including prion disease, AD, and ALS within brain myeloid cells, emphasizing the disease modulatory potential of this bacterium.

Microbiome-dependent transcriptional responses in the brain are dynamic. Our temporal analysis identified that immune cells and myelinating oligodendrocytes are acutely responsive to *E. coli* colonization, while neuronal populations do not display a response until 4 weeks post-colonization. Expression of those genes involved in adaptive immune pathways were initially robustly upregulated within microglia following colonization, but this response subsided by 4 weeks post colonization. However, the increase in immune pathways, particularly those involved in adaptive immunity, became apparent in nearly every other cell type by 4 weeks post colonization, suggesting that microglia are a focal point in relaying microbiome-derived signals to other cells in the brain. This solidifies prior observations of microbiome-dependent shifts in microglia state during both development and in models of neurodegenerative disease. It further suggests that microbiome-elicited microglia responses may trigger subsequent transcriptional impacts across other cell types in

the brain.

Colonization with *E. coli* was sufficient to induce a transcriptional response associated with neurodegenerative diseases in wildtype mice. We further observed that increased exposure to *E. coli* within the 5xFAD mouse model of AD accelerated the development of cognitive decline and amyloid pathology. Cognitive impairments were associated with decreased expression of genes within the classical DAM phenotype, thought to be an initially protective microglia state that limits the development and progression of AD outcomes(Jay, Hirsch et al. 2017, Keren-Shaul, Spinrad et al. 2017). This lack of protective response within brain myeloid cells may help to explain why disease outcomes progressed more rapidly in *E. coli* exposed mice. Interestingly, we and others, have observed that *E. coli* and related taxa within the *Enterobacteriaceae* family are sufficient to exacerbate pathologies in other models of neurodegeneration(Chen, Stribinskis et al. 2016, Sampson, Challis et al. 2020, Chongtham, Yoo et al. 2022, Liang, Liu et al. 2023). Our data herein expand on this concept, identifying specific brain transcriptional responses evoked by the presence of *E. coli* within the microbiome that may contribute to these outcomes. This is of particular importance given observations that *Enterobacteriaceae* are enriched in the gut microbiome amongst many neurodegenerative diseases(Fang, Kazmi et al. 2020).

While we utilized a model of AD-relevant pathologies, our data serve as a foundation to understand how microbiome-dependent transcriptional responses associated with specific microbial species can modulate neurological disease susceptibility. Systemic immune responses vary greatly in response to individual species, even within the same genera(Geva-Zatorsky, Sefik et al. 2017), suggesting that delineating individual microbial contributions is essential. While *E. coli* exemplifies how non-pathogenic microbiome derived signals shape the neurological transcriptional landscape, our data demonstrate that individual bacterial species—and perhaps bacterial strains—will induce differential outcomes. For example, we highlight the seemingly inverse consequences of *B. theta* on

neuroimmune transcriptional state compared to *E. coli,* yet it has been demonstrated that exposure to a related species of Bacteroides, *Bacteroides fragilis,* is sufficient to exacerbate AD outcomes(Cox, Schafer et al. 2019, Xia, Xiao et al. 2023, Wasén, Beauchamp et al. 2024). While both *Escherichia* and *Bacteroides* have been reported to be increased in people living with AD(Cattaneo, Cattane et al. 2017, Vogt, Kerby et al. 2017, Zhuang, Shen et al. 2018, Haran, Bhattarai et al. 2019, Liu, Wu et al. 2019, Khedr, Omeran et al. 2022, Wang, Li et al. 2022, Li, Cui et al. 2024), public studies of the AD-associated microbiome are currently limited. Understanding the species-specific associations in these human conditions and their experimental contributions to neurological functions remains an important gap in the field. Together, our results highlight the specificity and dynamics of microbiome-derived signals on the transcriptional landscape of the brain, as a foundation for the continued study of how the indigenous microbiome shapes overall brain health and disease susceptibility.

3.5. Methods

3.5.1. Experimental model and study participant details

3.5.1.1. Animals

Gnotobiotics. Germ-free (GF), male and female DBA/2N mice were originally obtained from Taconic Biosciences (#DBA2; RRID: IMSR_TAC:DBA2) following embryonic rederivation and bred within the Emory Gnotobiotic Animal Core (EGAC), for at least 3 generations prior to use in this study. GF animals were co-housed (with 2-5 same sex and age matched cage mates) in sterile cages within Parkbio rigid isolators. Mice were provided sterile food (Teklad Autoclavable Diet, 2019S) and water *ad libitum*. Microbiological testing (by culture and qPCR) was performed on all autoclaved materials entering isolators (including food, water, and bedding) as well as monthly within the isolators themselves. Prior to sacrifice or mono-colonization, all mice were transferred to

sterile, static housing in specific pathogen-free (SPF) vivarium. Mono-colonization was subsequently performed by oral gavage with ~10^8 cfu of bacteria of interest within a sterile, class II biological safety cabinet. Colonization status of all mice was confirmed by fecal culture at the time of sacrifice.

Conventionally-reared mice Conventionally-raised (CONV) male and female DBA/2J mice were originally obtained from Jackson Laboratory (#000671; RRID: IMSR_JAX:000671) and co-housed (2-5 per cage) in static housing with food (LabDiet: 5001) and water provided *ad libitum* within an SPF facility. Female and male 5xFAD mice, on a congenic C57Bl/6J background (Jackson Labs, #034848; MMRRC_034848-JAX) were maintained by crossing with C57Bl/6J wildtype mice (IMSR_JAX:000664). Mice were co-housed (2–5 per cage) with mixed genotype littermates in sterile, microisolator cages, under a 12h light/dark cycle with *ad libitum* access to sterile food (Teklad Autoclavable Diet, 2019S) and drinking water. Genotypes were confirmed with the following vendor-approved primers and their PCR parameters: APP Forward 5'-AGGACTGACCACTCGACCAG-3', APP Reverse 5'-CGGGGGTCTAGTTCTGCAT-3'; PS1 Forward 5'- AATAGAGAACGGCAGGAGCA-3', PS1 Reverse 5'-GCCATGAGGGCACTAATCAT-3'. All animal husbandry and experiments were performed in accordance with AVMA guidelines and approved by the Institutional Animal Care and Use Committee of Emory University (PROTO201900056).

3.5.1.2. Bacteria

Bacteroides thetaiotaomicron str. VPI 5482 (ATCC 29148), *Clostridium celatum* str. VPI 8759-1 (ATCC 27791), and *Lactobacillus johnsonii* str. VPI 7960 (ATCC 33200) were obtained from the American Type Culture Collection (ATCC). *Escherichia coli* str. K12 MC4100 (a kind gift from Matthew Chapman (University of Michigan(Sampson, Challis et al. 2020)) and *B. thetaiotaomicron* were grown aerobically at 37° C in tryptic soy broth (BD #211825) and brain heart infusion (BD

#237500) supplemented with hemin and vitamin K, respectively. *C. celatum* and *L. johnsonii* were grown anaerobically at 37° C in Chopped Meat Carbohydrate (Anaerobe Systems AS-811) and de Man-Rogosa Sharpe (BD 288130) broth respectively. Bacterial cultures were resuspended at ~10^8 cfu in sterile 50% glycerol and 5% sodium bicarbonate, plated to confirm monoculture, and stored at -80° C until use. Vehicle control consisted of sterile 50% glycerol (v/v) and 5% sodium bicarbonate (w/v) also stored at -80° C.

3.5.2. Method details

3.5.2.1. Bacterial enrichment

Male and female, conventionally raised 2-month-old 5xFAD and wildtype littermates were treated with an antibiotic cocktail consisting of 1mg/mL neomycin, 1mg/mL ampicillin, and 5mg/mL vancomycin in sterile water for 1 week. After antibiotic treatment, mice were randomly assigned to receive either ~10^8 cfu of *E. coli* by oral gavage 3x per week or vehicle (sterile 50% glycerol and 5% sodium bicarbonate) gavage. While cages consisted of mixed genotype animals, each cage of mice only received a single treatment to prevent cross-contamination. Each week, the colonization status of the mice was monitored by fecal culture.

3.5.2.2. Behavioral testing

Roughly equal numbers of male and female 5xFAD (n = 11-15) and wildtype (n= 9-23) littermates underwent behavioral testing after 1 month of enrichment, at 3 months of age. All behavioral testing was performed during the animal's light cycle. Before the start of any test, mice were habituated to the testing room in their home cage for 1hr. All behavioral tracking and analysis were performed using EthoVision XT software (Noldus Information Technology, Wageningen, the Netherlands) and the testing arenas/objects were cleaned between trials with 70% ethanol to

eliminate bacterial contamination and olfactory cues. Mice were tested on the following tests in order:

Open field test (OFT): As measures of motor and anxiety-like behavior, mice were placed in a 45cm square open field box for 10 minutes and allowed to explore freely. Distance traveled and time spent in the center (20x20cm) was recorded. The OFT also served as habituation for the object location test (OLT).

Object location test (OLT): Twenty four hours after the OFT, the OLT was run to assess short term spatial memory as described step-by-step in(Blackmer-Raynolds, Krout et al. 2024). The same open field box used in the OFT was used for the OLT, but additional landmarks (large papers with stripes, stars, or dots of different colors) were placed on 3 out of the four walls to allow for spatial orientation. During the initial study phase, mice were placed in the box with two identical copies of an object (either a plastic chess piece or 5mL Eppendorf tube) and allowed to explore freely for 10 minutes. The mice were then returned to their home cage for a 10-minute retention delay. During the testing phase, mice were returned to the box where one of the two objects had been moved to a novel location and allowed to explore freely for 5 minutes. Object exploration was considered time spent with the mouse's nose within 2cm of an object. Novelty preference was measured by taking the percent of total object exploration time that was spent exploring the moved object. A novelty preference significantly above 50% chance levels is indicative of intact memory as mice generally seek out the moved object.

Y-maze: In order to evaluate spatial working memory capacity, the Y maze test was performed as described in detail in(Blackmer-Raynolds, Krout et al. 2024). Mice were placed in a plastic Y shaped maze and allowed to explore freely for 8 minutes while the order of entries into each arm of the maze was recorded. Percent alternation was calculated by taking total number of

alternations (consecutive entries into 3 arms before repeating any arms) divided by the maximum possible alternations (total entries minus 2) and multiplying by 100. Mice with intact working memory display higher percent alternation as they prefer to explore previously unvisited areas.

Barnes maze: Longer term spatial learning and memory was evaluated using the Barnes maze test adapted from(Attar, Liu et al. 2013), and described in detailed in(Blackmer-Raynolds, Krout et al. 2024). Testing was performed over a 6-day period using a 92cm diameter, 20-hole, Barnes maze (MazeEngineers) with consistent extra-maze cues throughout. First, mice underwent a habituation trial in which they were placed on the maze with bright lights and white noise (66-70 dB) playing for 20 seconds then gently guided to an escape hole leading to a dark box filled with sterile bedding. After entering the escape box, the white noise was immediately turned off and the mice were allowed to acclimate to the escape box for 2 minutes. The mice then underwent 5 consecutive training trails spread over 2 days. During each training trial, mice were placed in the center of the maze (with white noise and bright lights on) and allowed to explore for up to 3 minutes. If the mice entered the escape box during this period, the noise was turned off and they were allowed to rest for 1 minute. Mice that did not enter the escape box after 3 minutes were gently guided to the proper hole before being allowed to rest. The probe trial occurred 72hr after completion of the final training trail. During the probe trial, the escape box was removed, and mice were placed on the maze (lights and noise on) to explore for 90 seconds. During both training and probe trials, primary latency (the time it took for the mouse to first check the escape hole) was recorded by hand.

Intestinal behaviors: To evaluate whether colonization disrupts gastrointestinal (GI) function, a subset of roughly equal male and female mono-colonized (n=6-15) and 5xFAD mice (n=5-9) underwent a set of GI tests. First, colonic transit was measured by fecal output testing described in detail in(Hamilton, Krout et al. 2024). Fecal output testing was performed within a level II biosafety cabinet within the animal's vivarium with no prior habituation period. Mice were placed in individual

sterile 1L plastic beakers for 30 minutes and the number of fecal pellets produced was recorded every 5 minutes. Fecal water content was measured by collecting the fecal pellets produced during the fecal output test and weighing them before and after drying at 100°C for 48hr. As a measure of total intestinal transit time, carmine red dye elution was performed as described in(Hamilton, Krout et al. 2024). Testing was performed in the behavioral testing room. Mice were allowed to acclimate for 1hr before undergoing oral gavage with 100µl of sterile carmine red dye (6% w/v) (Sigma, C1022) dissolved in 0.5% methylcellulose (w/v; Sigma, M7027). Mice remained in their home cages for 2 hours then were transferred to individual sterile cages devoid of bedding, but with access to a small amount of sterile food and water. The cages were observed every 15 minutes for the presence of a red fecal pellet. Once a red pellet was discovered, the time was recorded, and the mouse was returned to its home cage.

3.5.2.3. Tissue collection and processing

Mice were humanely euthanized via open-drop isoflurane overdose followed by cardiac puncture to collect blood samples, perfusion with PBS, and exsanguination. To collect serum, blood was immediately placed into vacuette collection tubes (Griener #454243) and spun at room temperature at 1,800 g for 10 minutes. Serum was then stored at -80° C until use. Brain tissue was either A) immediately processed by immunopanning or magnetic activated cell sorting (MACS; see below); B) dissected and flash frozen in liquid nitrogen and stored at -80° C for later protein or nuclei isolation; or C) fixed for 24hr in 4% paraformaldehyde and stored at 4° C in sodium azide for immunohistochemistry. Intestinal tissue was removed from the mouse and colon length was measured as a marker of overall intestinal inflammation. Approximately 1cm of tissue was dissected from the ilium and proximal colon, flash frozen in liquid nitrogen and stored at -80° C for multiplex ELISA.

3.5.2.4. Brain myeloid cell enrichment

Immediately after sacrifice, brains were homogenized using a Wheaton dounce tissue grinder (with 0.114 ±0.025mm pestle clearance) in culture media comprised of HBBS with 1.5% HEPES, and 0.5% glucose. Cells were then transferred to a 35% isotonic percoll gradient solution and spun 20 minutes at 700 x g to remove the myelin layer. Transcription (Actinomycin D, Sigma #A1410) and translation (Anisomycin, Sigma #A9789) inhibitors were added to every solution throughout both procedures to prevent transcriptional changes that could impact downstream results. The remaining cells were washed several times in HBBS culture media before undergoing either immunopanning or magnetic activated cell sorting (MACS) separation.

Immunopanning. Cells were resuspended in 0.02% BSA in PBS and incubated at room temperature on a 10cm petri dish with anti-CD11b and secondary antibody already adhered to the dish (Thermo Scientific, 14-0112-82 and Jackson 112-005-167) for 10-15 minutes. After incubation the unadhered cells were washed away and remaining cells scraped off the plate directly into Trizol (Zymo Research R2050-1-200) and stored at -80° C until later use.

MACS. Sorting was performed according to the manufacturers (Miltenyi Biotec) guidelines. Samples were resuspended in PB buffer (0.5% BSA in PBS pH 7.2) with CD11b MicroBeads (Miltenyi Biotec # 130-093-634) and incubated for 15 minutes at 4° C protected from light. Cells were then washed and resuspended in PB buffer before being placed on an LS column (Miltenyi Biotec #130-042-401). Magnetic separation was repeated on a second LS column to maximize purity. After separation, cells were resuspended in Trizol and stored at -80° C for later use.

3.5.2.5. Single nucleus preparation and sequencing

Flash frozen hippocampal samples from 12–15-week-old female germ-free, conventionally

raised, and mono-colonized mice (n = 4) underwent a nuclei isolation protocol adapted from(Corces, Trevino et al. 2017). Frozen tissue was placed in homogenization buffer (consisting of 0.26M sucrose, 0.03M KCl, 0.01M $MgCl_2$, 0.02M Tricine-KOH pH 7.8, 0.001M DTT, 0.5mM Spermidine, 0.05mM Spermine, 0.3% NP40, and protease inhibitor) and homogenized using both pestle A (0.0030-0.0050 in. clearance) and pestle B (0.0005-0.0025 in. clearance) of a KIMBLE dounce tissue grinder (Sigma D8938). Density gradient centrifugation was performed using iodixanol and the nuclei band was captured at the 30%-40% iodixanol interface. Nuclei were washed in RSB buffer (0.01M Tris-HCL pH 7.5, 0.01M NaCl, 0.003M $MgCl_2$, and 0.1% Tween-20) then samples were combined such that each treatment group was represented by two samples, each containing roughly equal numbers of cells from two mice within a single treatment group. Then, samples immediately underwent cell capture using the Chromium Next GEM Single Cell 3' Kit v3.1 (10x Genomics # PN-1000268) according to the manufacturer's guidelines. Approximately 1,600 cells were loaded onto a Chromium Next GEM Chip and run using a Chromium Controller and library preparation steps were performed according to the manufacturer's guidelines. Pooled samples were sequenced on a NovaSeq X Plus 25B 2x150 for 5-6.25B PE reads total by Admera Health (South Plainfield, NJ).

3.5.2.6. Single nucleus RNA-seq analysis

Reads were aligned to the mouse reference genome (GRCm39) using the Cell Ranger pipeline (version 4.0.10, 10x Genomics). The Cellbender(Fleming, Chaffin et al. 2023) (version 0.3.0) remove-background function was used to minimize the effects of ambient RNA (expected cells=5,500 and total droplets included=12,000). The snRNA-seq libraries were imported into R (version 4.3.2) using Seurat(Hao, Stuart et al. 2024) (version 5.1.0) then filtered to only include cells with 200-7,500 features and less than 6% mitochondrial reads. Data was normalized, scaled, and

clustered using Seurat defaults and doublets were removed using DoubletFinder(McGinnis, Murrow et al. 2019) (version 2.0.4) with a multiplex rate of 0.8% per 1,000 cells. Samples where integrated using Seurat RPCA Integration then re-clustered to create an integrated UMAP. Seurat "FindAllMarkers" function was used (min.pct = 0.25, thresh.use = 0.25) to identify marker genes for each cluster. Manual cell type annotation was performed by identifying cell types with high marker gene expression within the Allen Brain Cell Atlas(Alzheimer's Association). Differential expression analysis was performed within each cell cluster of interest by using MAST within the Seurat "FindMarkers" function. Genes were considered differentially expressed if they had an absolute log fold change value greater than 1 and adjusted p value of less than 0.001. Functional annotation clustering was performed using Metascape(Zhou, Zhou et al. 2019) (version 3.5.20240901) with all DEGs except genes without a canonical name (those ending in "rik" or beginning with "Gm") and GSEA was run using clusterProfiler(Wu, Hu et al. 2021) (version 4.10.1).

3.5.2.7. Bulk RNA-seq preparation and sequencing

RNA was extracted from isolated brain myeloid cells using the Qiagen RNeasy Kit (#74106) according to the manufacturer's guidelines. Bulk RNA-sequencing libraries were created for each sample using the Takara SMART-Seq mRNA HT LP kit (#634792) according to the manufacturer's protocol and recommendations. Sequencing was performed by Admera Health (South Plainfield, NJ) using a NovaSeq or HiSeq to achieve approximately 85 million total reads per sample.

3.5.2.8. Bulk RNA-seq analysis

Sequencing quality control was performed using FastQC (version 0.11.9) and reads were pseudoaligned to the mouse reference genome (GRCm39) using Kallisto (version 0.44.0)(Bray, Pimentel et al. 2016). Files were imported into R (version 4.3.2) and counts were converted into TMM normalized $\log_2$ counts per million. Genes expressed in less than 3 samples were excluded

from the analysis. Differential gene expression analysis was performed using limma(Ritchie, Phipson et al. 2015) (version 3.58.1) and edgeR(Robinson, McCarthy et al. 2010) (version 4.0.16). Genes were filtered to only include genes enriched in microglia compared to other brain cell types based on expression levels published by the Brain RNA-seq Website(Zhang, Chen et al. 2014). Genes were considered differentially expressed if they had a Bonferroni adjusted p value less than 0.05. Functional annotation clustering was performed using DAVID(Huang, Sherman et al. 2009, Sherman, Hao et al. 2022) and clusterProfiler(Wu, Hu et al. 2021) (version 4.10.1), gene set enrichment analysis was performed by fgsea(Korotkevich, Sukhov et al. 2016) (version 1.28.0).

3.5.2.9. Protein extraction and ELISA

Protein was extracted from flash frozen brain and gut samples by sonication in homogenization buffer consisting of 125mM Tris, 15mM $MgCl_2$, 2.5mM EDTA (PH 7.2), 1% Triton X 100, and protease inhibitor (Roche #11697498001, 1 tablet per 10mL). Samples were centrifuged at 20,800 rcf for 10 minutes at 4° C. The protein concentration of the supernatant was measured using a Pierce BCA Protein Assay Kit (Thermo #23225) according to the manufacture's guidelines. When quantifying amyloid beta levels, a three part protein solubility protocol outlined in(Blackmer-Raynolds and Sampson 2024) was performed instead. First, the tris soluble fraction was isolated by sonication in 125mM Tris, 15mM $MgCl_2$, 2.5mM EDTA (PH 7.2), and protease inhibitor (Roche #11697498001, 1 tablet per 10mL). Following centrifugation, the pellet was then re-sonicated in the above buffer with the addition of 1% Triton X 100 to isolate the triton soluble fraction. Finally, the remaining pellet was sonicated in buffer containing 70% formic acid to extract the most insoluble protein fraction. Protein samples as well as pure serum samples were then run using multiplex ELISA (Meso Scale Discovery V-PLEX Proinflammatory Panel 1 (mouse) Kit # K15048D and V-PLEX human amyloid beta peptide kit #K15200E).

3.5.2.10. Immunohistochemistry

Paraformaldehyde fixed brain hemispheres were transferred to a 30% sucrose solution for 24-48hr then frozen in Tissue-Tek O.C.T. compound (#4583) and sliced into 40μm coronal sections using a cryostat. Two to four sections representing the anterior hippocampus including the 3rd and lateral ventricles were stained per mouse. Antigen retrieval was performed prior to IBA1 staining by heating the tissue to 90° C for 5 minutes in a sodium citrate antigen retrieval buffer (10 mM tri-sodium citrate dihydrate and 0.43mM Tween 20, pH 6.0). Tissue was then blocked in 1% BSA, incubated in anti-IBA1 antibody (Wako #019-19741 1:1,000), and anti-rabbit 594 secondary (Thermo #A11012, 1:1,000). Imaging was performed using a Keyence BZ-X Series microscope (Itasca, IL) at 20X magnification. Images were processed in Fiji using a macro created by the Emory Integrated Cellular Imaging Core that auto thresholded the images using "RenyiEntropy dark," converted to mask, and calculated IBA1% area within a hand traced region of interest around the hippocampus. MHCII, CD31, and CD206 staining was performed without antigen retrieval using the following antibodies and concentrations: MHCII (BioLegend #107602) 1:300 with anti-rat biotin (Thermo #A187843) and streptavidin 488 (Thermo #S31354); CD31(R&D Systems # AF3628) 1:400 with anti-rabbit 594 (Thermo #A21207) 1:1,000; and CD206 (Cell Signaling # 24595T) 1:400 with anti-goat 647 (Thermo #A21447). A streptavidin/biotin blocking kit (Vector labs SP-2002) was used in conjunction with biotinylated antibodies to improve MHCII signal. Images were taken at 20x using a Leica SP8 multiphoton microscope and analyzed using Fiji "Li" and "Moments" auto thresholding for MHCII and CD206 respectively. Microscopy and image analysis was performed by a blinded lab member.

3.5.3. Overview of statistical tests

Unless otherwise indicated in the figure legends, data are expressed as mean ± SEM. Sample

sizes are indicated in the figure legends, and when possible, each individual sample is represented as its own point on each graph. In all analyses except snRNA-seq, a sample represents an individual mouse. In SnRNA-seq a single sample represents combined data from two mice of the same treatment. Statistical tests for all non-transcriptomic data were performed using GraphPad Prism 8. One-way ANOVAs were used to compare mono-colonized mice and T tests were used to compare enriched (5xFAD and wildtype) mice. The object location test was analyzed by a one sample T test comparing each treatment to the 50% chance level. Unless otherwise noted significance was determined to be a p value of less than 0.05. Details on transcriptomic data analysis can be found in their respective sections above. All unique code is publicly available on GitHub upon acceptance.

3.6. Acknowledgments

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3.7. Figures

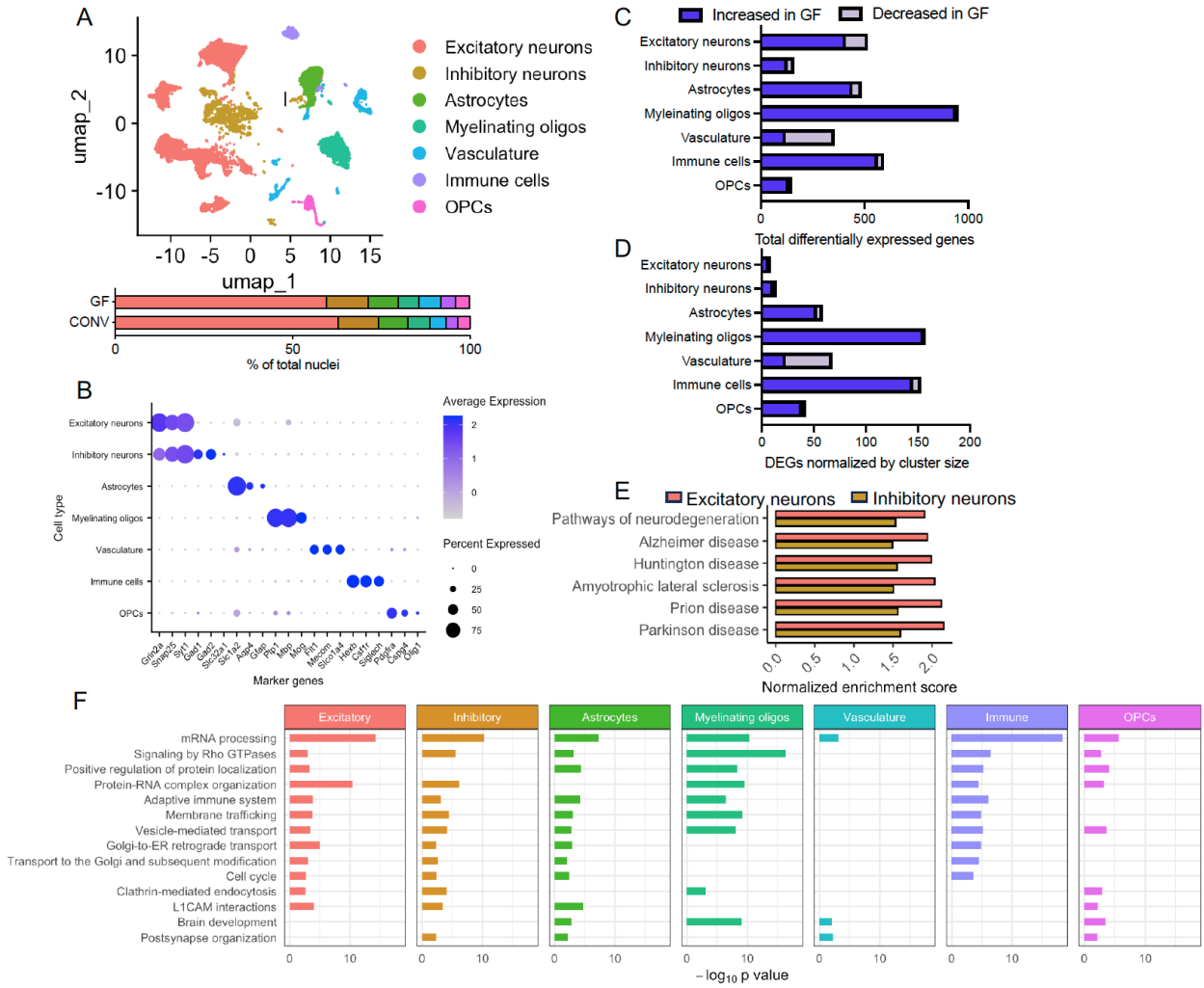


Figure 3.1. The microbiome shapes the transcriptional landscape of every major cell type in the brain. A) Single nucleus RNA-seq was performed on hippocampal samples from female germ-free (GF) and conventionally raised (CONV) mice. UMAP shows all major cell types identified as well as the amount of each cell type found within each treatment group. B) Cell type markers representing each major cell type cluster. C) Total number of differentially expressed genes (DEGs; log fold change $> |1|$, $p < 0.001$) in GF mice compared to CONV across each cell type. D) DEGs normalized by the number of cells in that cluster. E) Gene set enrichment analysis showing increased neurogenerative disease KEGG pathways in GF mice. F) Representative pathways increased in at least four cell types by overrepresentation analysis (Metascape). Data represents cells from 4 mice per treatment.

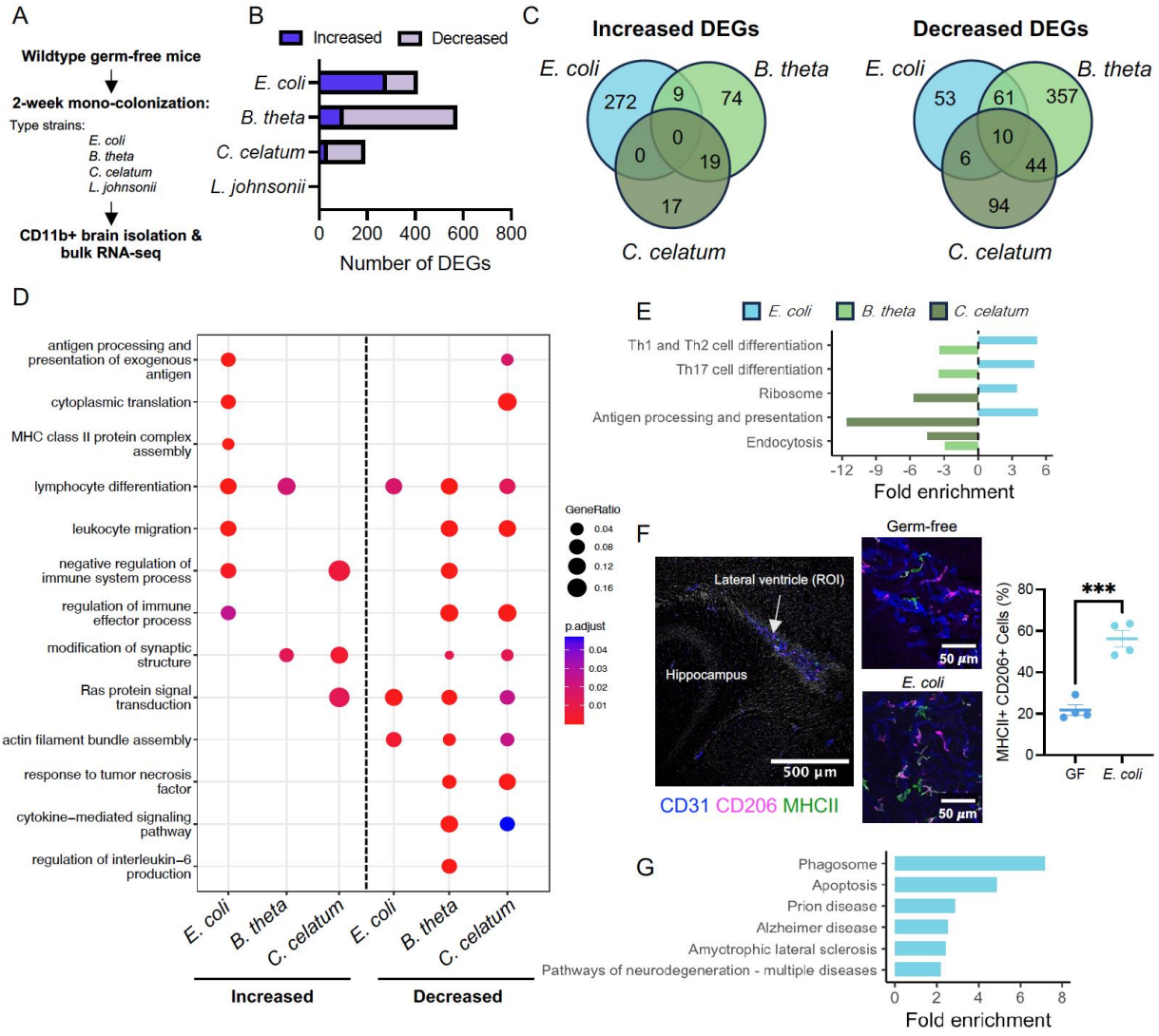


Figure 3.2. Select gut bacteria uniquely shape neuroinflammatory tone. **A)** Wildtype germ-free (GF) mice were mono-colonized with type strains of interest for 2 weeks. After mono-colonization, CD11b+ myeloid cells were isolated from the brains and bulk RNA-seq was performed. **B)** Number of differentially expressed genes (log fold change > |0.25|; $p < 0.05$) in mono-colonized mice compared to GF. **C)** Number of overlapping differentially expressed genes across colonization states. **D)** Comparison of overrepresentation-based pathway analysis results across mono-colonization states. **E)** KEGG pathway analysis was run on both increased and decreased DEGs using DAVID. KEGG pathways significantly increased or decreased in at least two treatments are shown. **F)** Immunohistochemistry was performed on the choroid plexus of the lateral ventricle to quantify the percent of MHCII+ CD206+ double-positive CD206+ cells. **G)** KEGG pathways increased after *E. coli* mono-colonization. n = 3-7 mice. In **F)** dots represent individual mice and error bars represent SEM. *** $p < 0.001$.

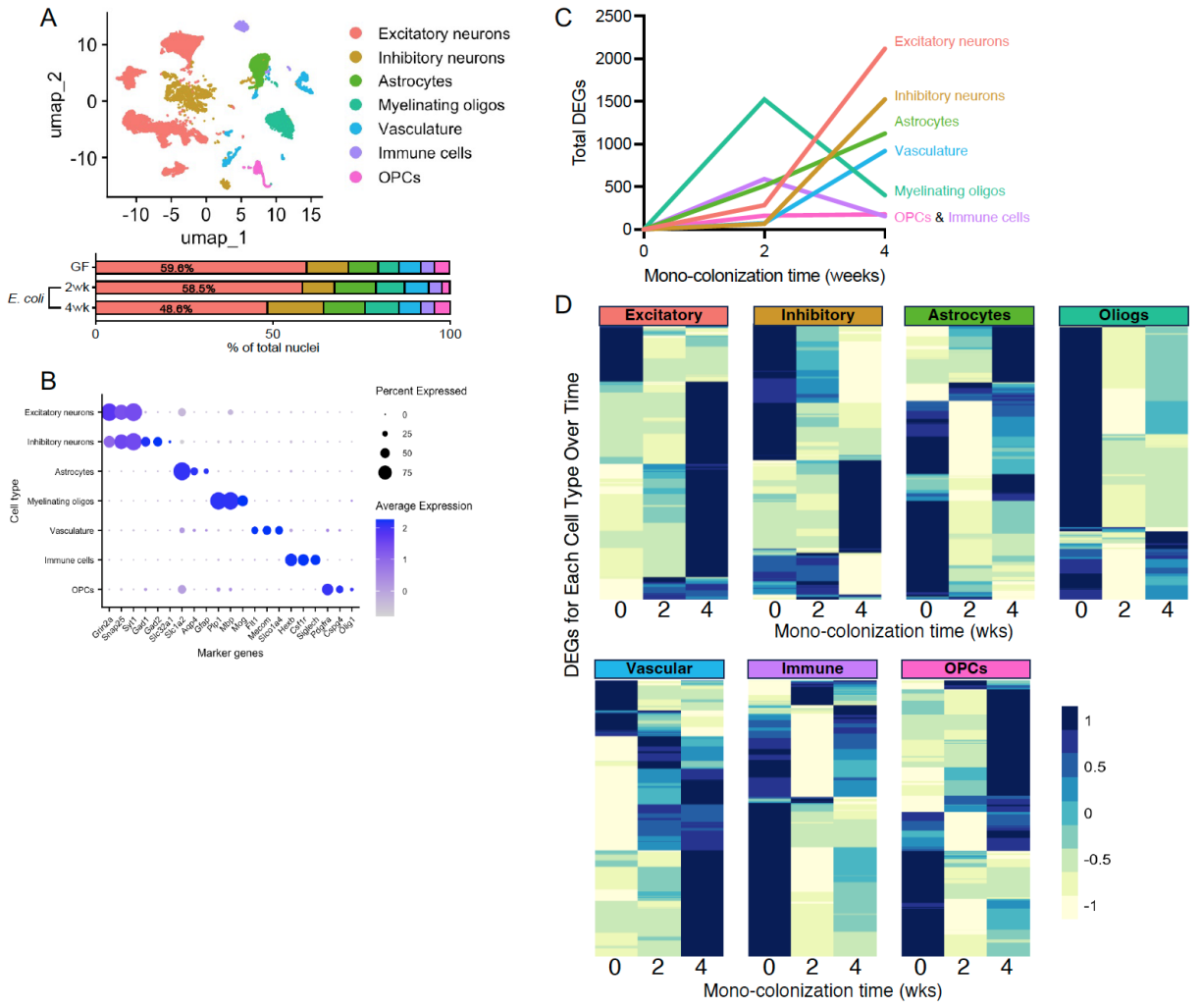


Figure 3.3. Gut colonization by *E. coli* temporally modulates the gene expression of all cell types the brain. Single nucleus RNA sequencing was performed on hippocampal samples from germ-free (GF) mice and mice that were mono-colonized with *E. coli* for either 2 or 4 weeks. A) UMAP showing cell clusters and bar graph showing the percentage of total nuclei within each cluster based on treatment. B) Cell type markers for each major cell type cluster. C) Graph showing the total number of differentially expressed genes (DEGs; log fold change > |1|, adjusted $p < 0.001$) compared to GF per cell type across time. D) Heat map shows relative expression levels of all differentially expressed genes (at either 2 or 4 weeks) within each cell type across time (note each heat map represents only that cell type's DEGs). Data represents cells from 4 mice per treatment.

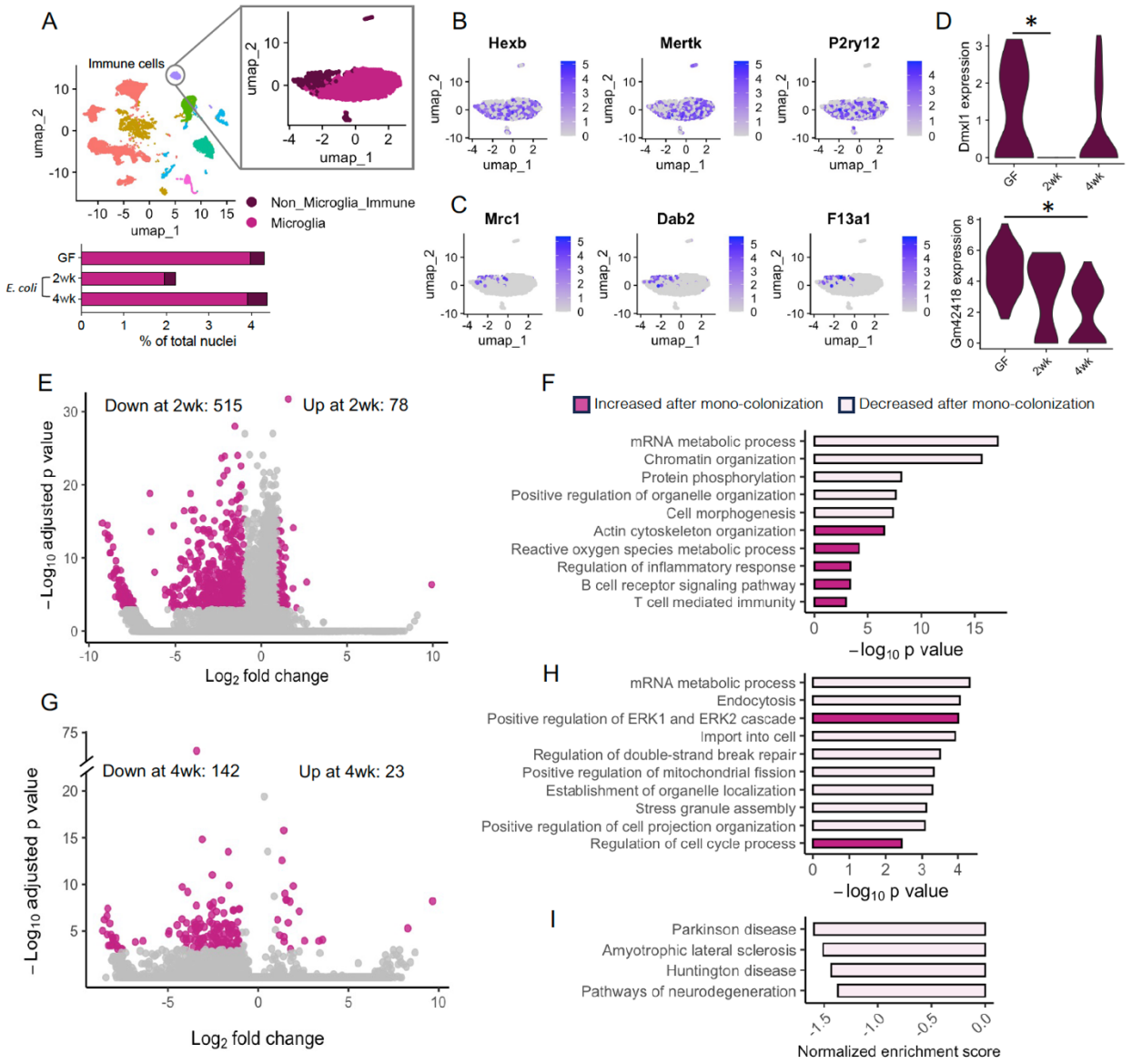


Figure 3.4. *E. coli* colonization induces temporal regulation of microglial transcriptional state. The immune cell cluster identified in Figure 3.2 was further subclustered to distinguish microglia from non-microglia immune cells. A) Shows cell clustering UMAP and % of all nuclei within each cell type. Markers defining each cell cluster are overlayed upon the immune UMAP with B) representing microglia markers and C) representing non-microglia markers. Differential gene expression analysis was performed only both cell clusters. D) shows violin plots of the two DEGs that reached significance in the low powered non-microglia immune cluster. E) Volcano plot and F) pathway analysis of microglia differentially expressed genes (log fold change > |1|, $p < 0.001$) after 2 weeks of *E. coli* mono-colonization. G) Volcano plot, H) GO pathway analysis and, I) neurodegeneration KEGG GSEA of microglia after 4 weeks of mono-colonization. Cells represent 4 mice per treatment.

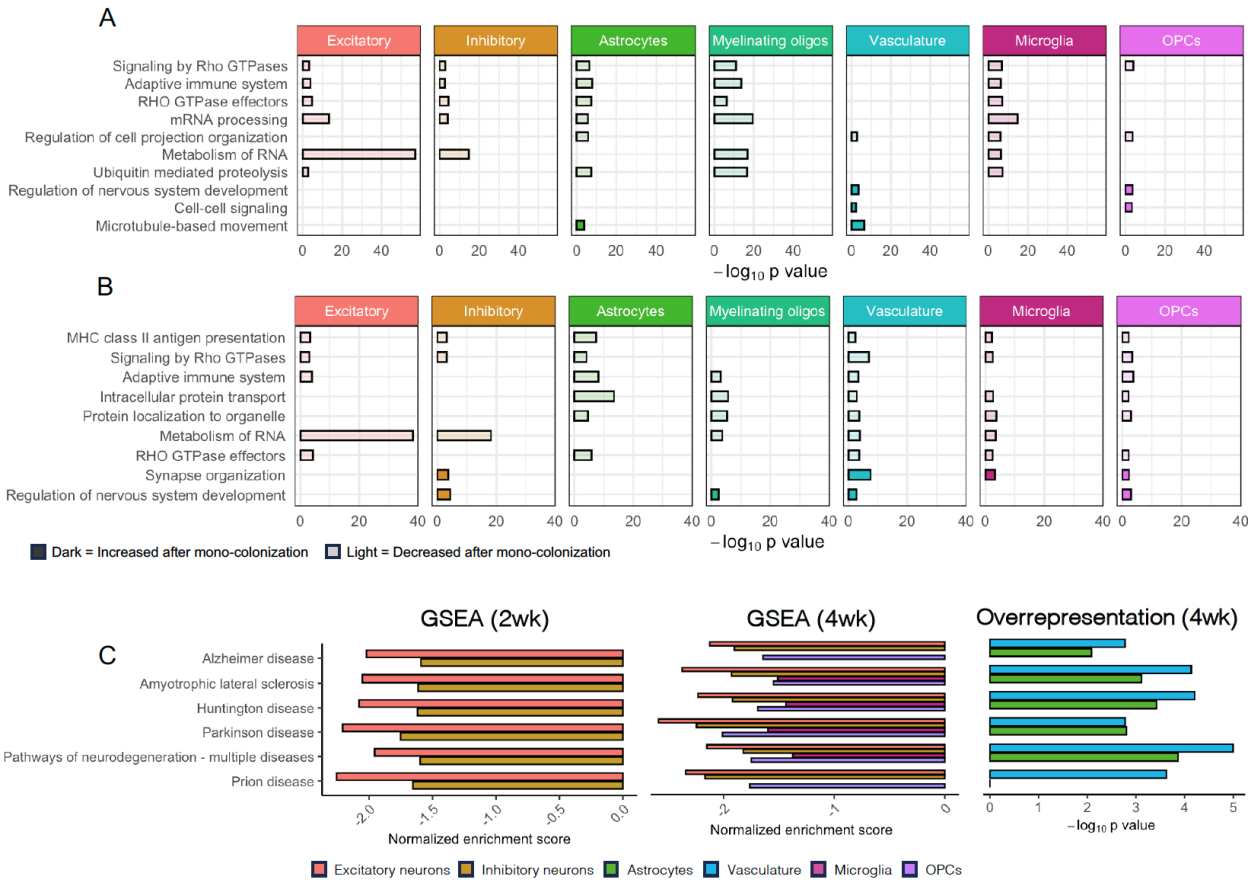


Figure 3.5. *E. coli* mono-colonization modulates biological pathways involved in adaptive immune responses and neurodegeneration across cell types and timepoints. Overrepresentation pathway analysis was run using the DEGs (log fold change > |1|, p value < 0.001) between GF and *E. coli* mono-colonized mice at each timepoint. Increased and decreased pathways that overlapped the most across cell types at 2 weeks are shown in A) and at 4 weeks are shown in B). Gene set enrichment analysis was also performed to identify neurodegeneration KEGG pathways that are modulated by *E. coli* colonization. C) Shows the normalized enrichment score for each cell type with a significant GSEA result at 2 and 4 weeks as well as neurodegeneration KEGG pathways increased in overrepresentation analysis at 4 weeks. Data is from 4 mice per treatment.

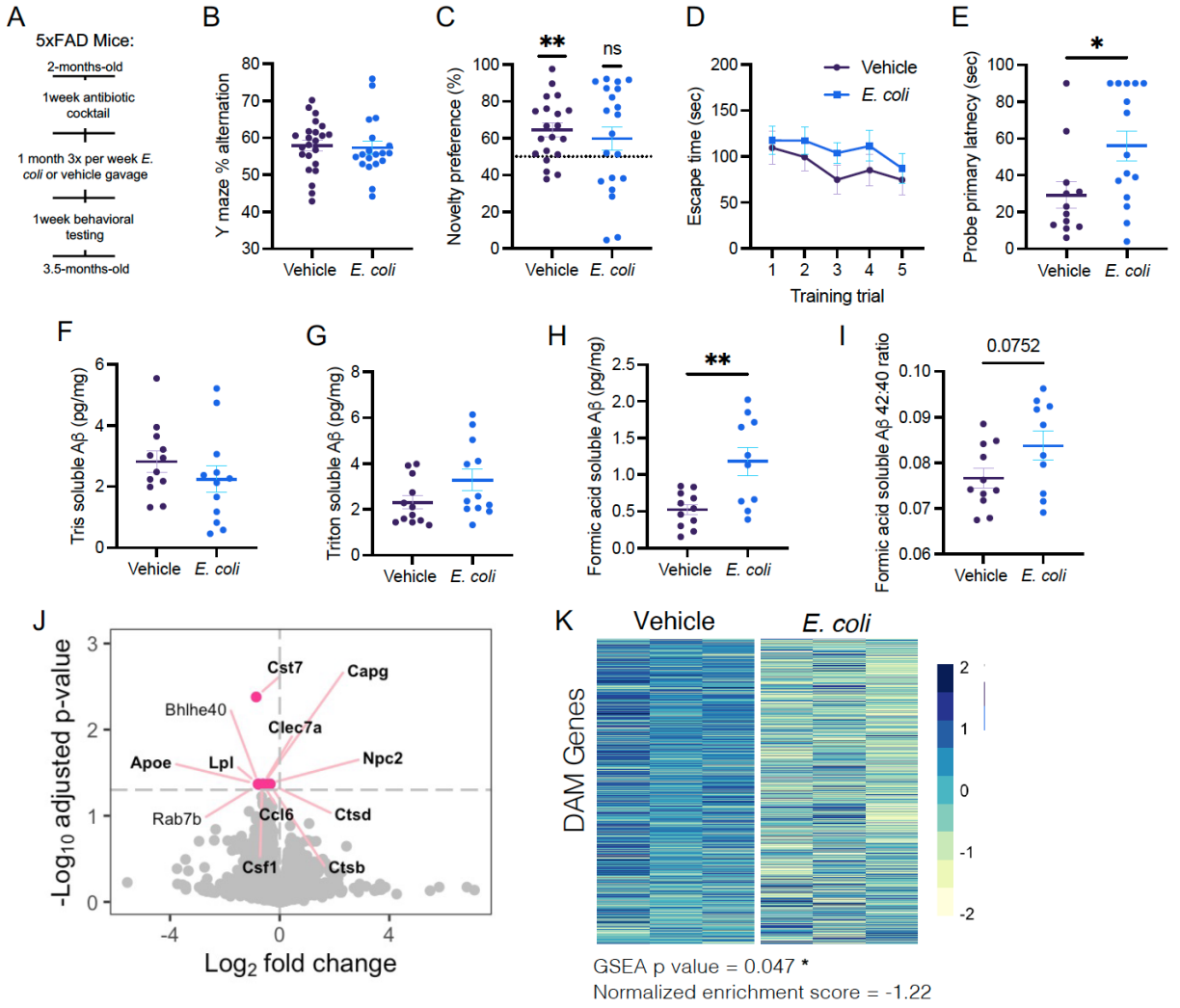
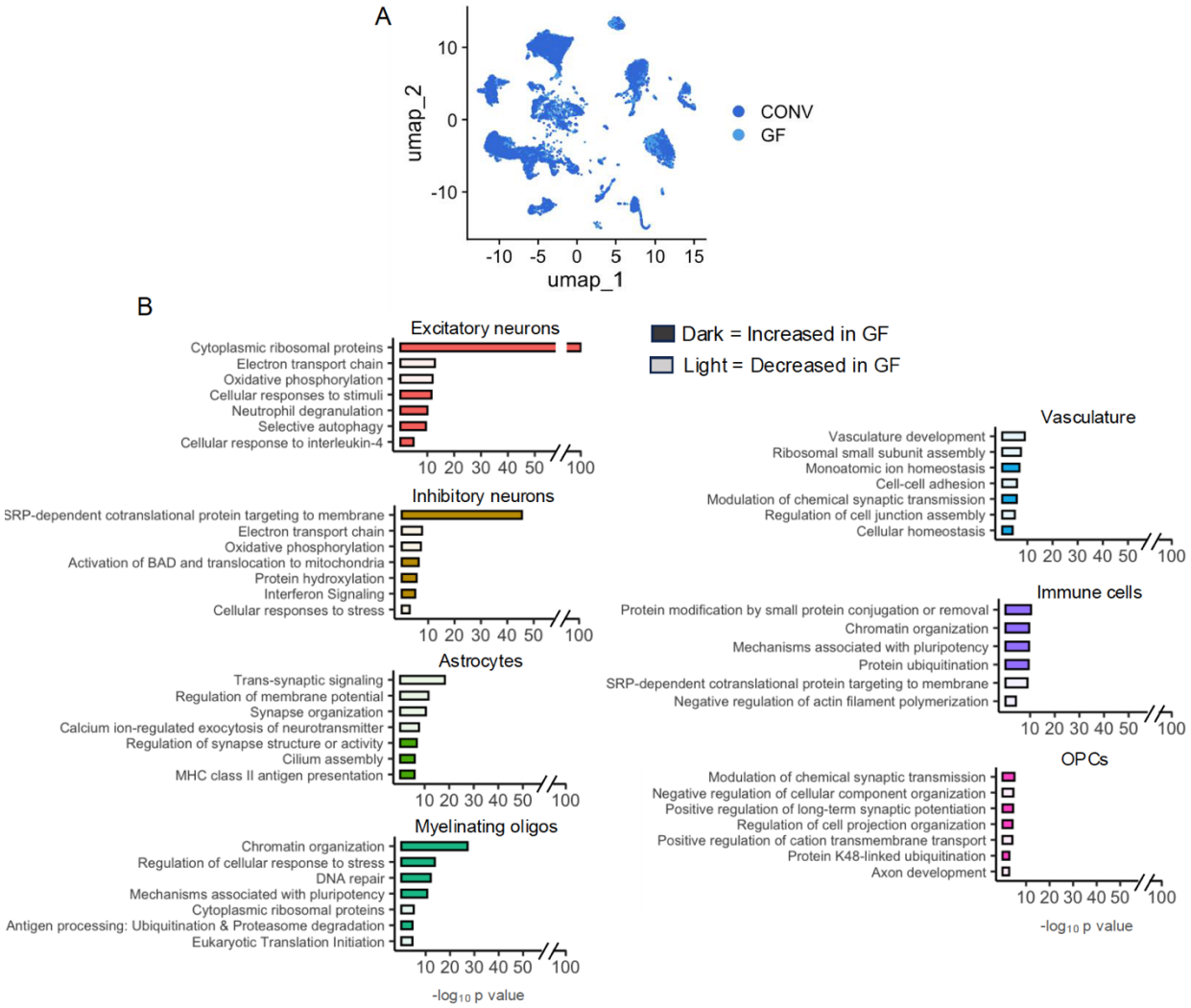
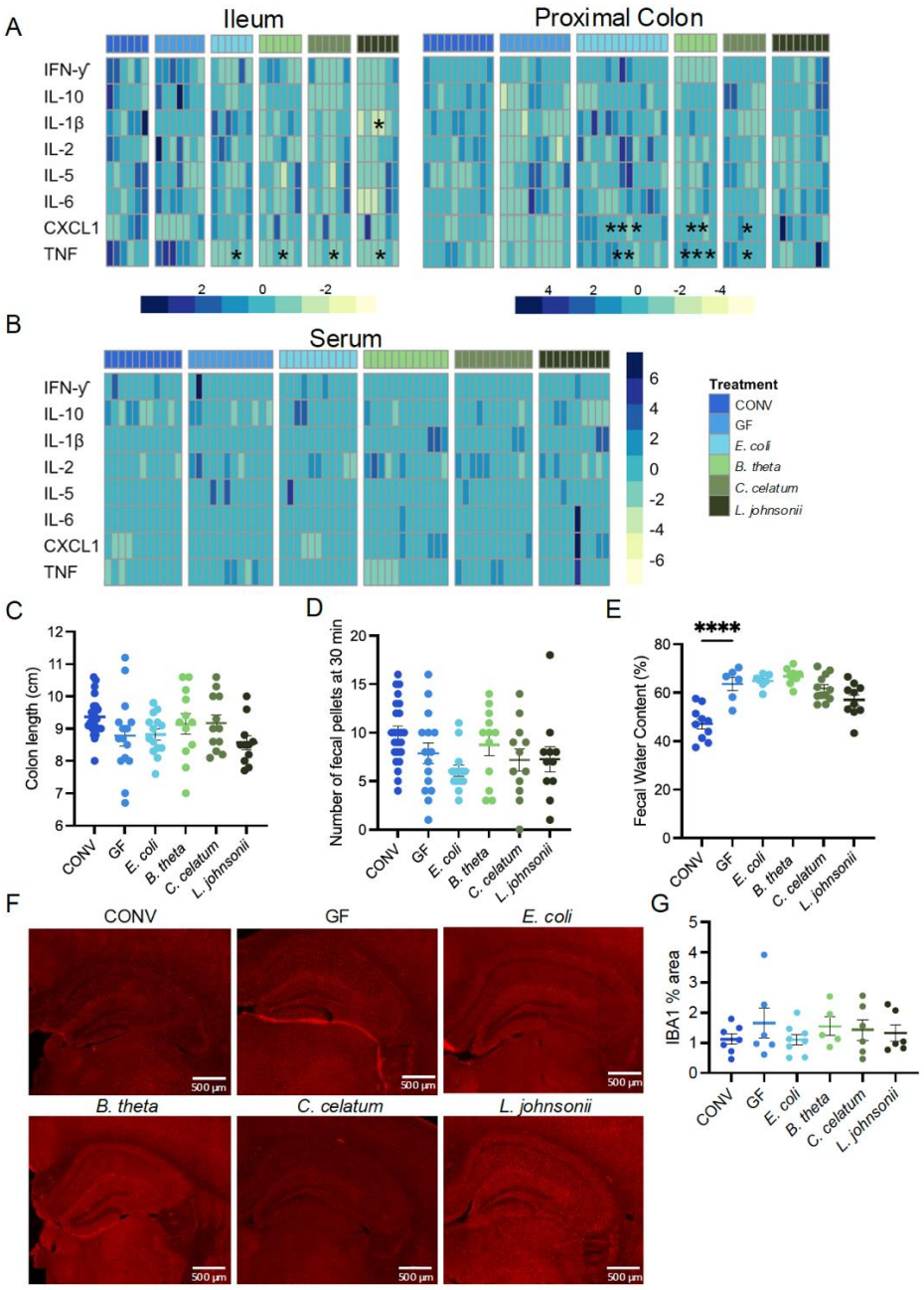


Figure 3.6. *E. coli* exposure exacerbates AD outcomes in 5xFAD mice. A) Male and female 2-month-old 5xFAD mice underwent a 1-month enrichment paradigm followed by behavioral and pathological assessments. Behavioral testing was performed on the B) Y maze, C) Object location test, and D-E) Barnes maze to evaluate different forms of spatial memory function. Levels of amyloid beta were measured within F) tris soluble, G) triton soluble, and H) formic acid soluble protein fractions extracted from the hippocampus. I) The ratio of amyloid beta 42:40 as also calculated the formic acid soluble fraction. Bulk RNA-seq was performed on CD11b+ myeloid cells in the brain with J) showing a volcano plot of differentially expressed genes with disease associated microglia (DAM) genes highlighted in bold and K) showing the expression level of all DAM genes as well as results of a gene set enrichment analysis (GSEA). For B - I n = 11-15, dots represent individual mice, bars represent mean ± SEM. Groups were compared using a two tailed t test except C where each condition was compared to the 50% chance level using a one sample t test. J-K) n = 3 dots (J) and rows (K) represent individual genes and columns represent individual mice (K). * $p < 0.05$ ** $p < 0.01$.

3.8. Supplementary Figures

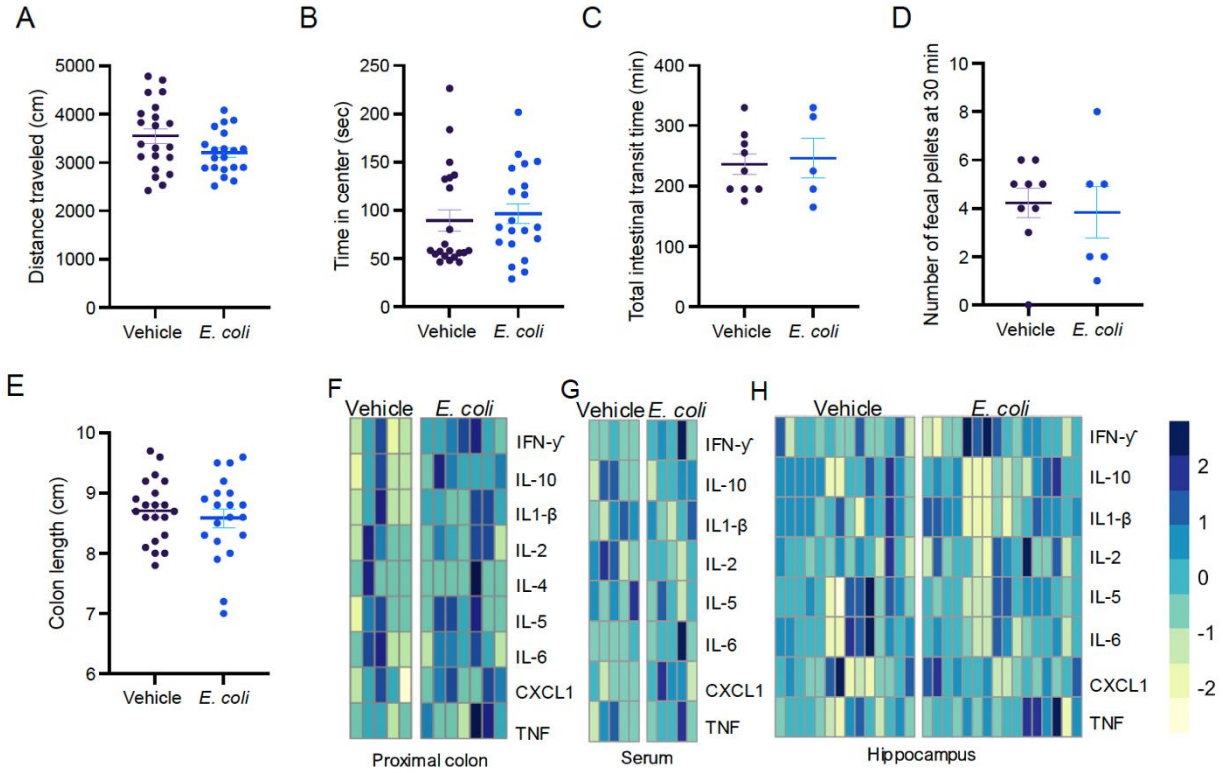


Supplementary Figure S3.1, *associated with Fig. 3.1.* Pathways altered in GF mice by cell type. A) Hippocampal single-nucleus RNAseq, as in Fig. 1, UMAP with nuclei highlighted by colonization status. **B)** Overrepresentation based pathway analysis was run on every major cell cluster using Metascape (log fold change > |1|, $p < 0.001$). Representative pathways that are increased and decreased in each cell type (and not included in Fig. 1E) are shown. Cells are from 4 mice per treatment group.

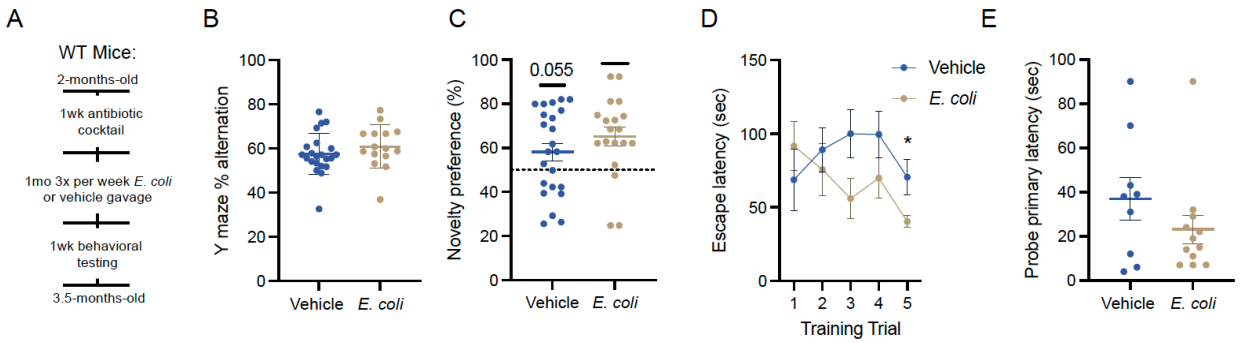


Supplementary Figure S3.2, *associated with Fig. 3.2*. Neurodegeneration-associated gut bacteria modulate intestinal, but not circulating cytokine levels or microgliosis. **A)**. Inflammatory cytokines and chemokines were measured in the ileum, proximal colon, and **B)** serum by multiplex ELISA. **C)** Colon length at the time of sacrifice was recorded as a measure of generalized colonic inflammation. **D)** Fecal output and **E)** fecal water content was used to assess gastrointestinal function. **F)** IBA1 staining was performed in the hippocampus and **G)** % area was compared between groups. n = 5-24. Dots (graphs) or columns (heat maps) represent individual mice. Error bars represent mean ± SEM. All treatment groups were compared to germ-free (GF) using either two-way repeated measures ANOVAs (**A-B**) or one-way ANOVAs **(C-E, G**) with Dunnett's multiple comparison tests**.** * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to GF. Conventionally colonized (CONV).

Supplementary Figure S3.3, *associated with Fig. 3.5. E.* coli modulates unique biological pathways for each cell type at 2 and 4 weeks. Overrepresentation based pathway analysis was run on the increased and decreased DEGs for each cell type (log fold change > |1| , p <0.001) after 2 and 4 weeks of mono-colonization using Metascape. Representative pathways (excluding those shown in Figure 5) for the 2-week timepoint are shown in **A)** and 4-week timepoint are shown in **B).** Data is from 4 mice per treatment.



Supplementary Figure S3.4, *associated with Fig. 3.6. E. coli* exposure does not induce sickness behavior or robust inflammation in 5xFAD mice. Vehicle and *E. coli* exposed 5xFAD mice were tested for signs of sickness behavior on a battery of tests. **A)** Motor and **B)** anxiety-like behavior were measured on the open filed test. Gastrointestinal function was measured by **C)** carmine red elution and **D)** total fecal output during a 30-minute period. Inflammation was measured within the colon via **E)** colon length at the time of sacrifice and inflammatory cytokines and chemokines were measured within the **F)** proximal colon, **G)** serum, and **H)** hippocampus by multiplex ELISA. n = 5-21. Dots (or columns in **F-H**) represent individual mice, bars represent mean ± SEM. In **A-E** groups were compared using a two tailed t test. In **F-H,** groups were compared using multiple t tests adjusted for multiple comparisons using two-stage step-up (Benjamini, Krieger, and Yekutieli).



Supplementary Figure S3.5, *associated with Fig. 3.6. E. coli* exposure does not induce cognitive impairment in wild type mice. A) Wild type littermates were tested side by side with 5xFAD mice to see if *E. coli* exposure was sufficient to induce cognitive decline in the absence of familial AD mutations. Performance on the **B)** Y maze, **C)** object location test and **D-E)** Barnes maze. n = 9-23. Dots represent individual mice bars represent mean ± SEM. Groups were compared using a T test in **B** and **E**, one sample T test compared to 50% chance level in **C**, and two-way repeated measures ANOVA in **D**. *$p < 0.05$; ** $p < 0.01$.

Chapter 4: Discussion

4.1 Implications for the field

The present dissertation research highlights the critical role of microbial signals in shaping neural health across brain cell types and in both health and disease contexts. To our knowledge, this study represents the first comprehensive single-cell dataset simultaniously highlighting the effects of microbial signals in all major brain cell types. We emphasize that the consequences of the microbiome are not isolated to a single cell type or biological pathway, but are instead incredibly diverse and wide-sweeping. In our analysis, we present an overview of some of these findings, highlighting the relevance of microbial signals for neuroimmune and neurodegenerative disease pathways throughout the brain, however, our analysis only brushes the surface of the available data. From this dataset, it is clear that microbial signals modulate a vast array of biological processes that could be relevant across all neurological disease contexts. My hope is that researchers will each look at this dataset from their own unique perspective, allowing for continued discoveries to be made and an increased awareness of the important effects of microbial signals on the brain in all new contexts.

In addition to emphasizing the importance of microbial signals as a whole, the present study also underscores the unique effects of individual bacteria on neuroinflammatory tone. Where many microbiome studies manipulate the entire microbial community, our reductionist mono-colonization approach allows us to pinpoint the specific effects of single microbes on the brain. Through this work, we highlight the unique and sometimes even opposite effects of individual microbes on neuroinflammatory tone, emphasizing the importance of studying individual bacterial taxa rather than just entire microbial communities. We demonstrate that some microbes, such as *L. johnsonii,* have no overt impacts on neuroinflammatory tone, whereas bacteria such as *E. coli* have robust consequences that translate to changes in Alzheimer's disease (AD) outcomes. It is therefore only through this

reductionist mono-colonization approach that we can begin to identify potential disease-modulatory bacteria that can act as therapeutic targets.

4.2 Limitations in the identification of an Alzheimer's disease-associated microbiome

In order to truly understand how specific microbes may be contributing to AD outcomes, it is essential that we first understand which microbes to study. While every attempt was made to select bacteria that represent taxa that are altered in patients with neurodegenerative disease, the selection process was severely hampered by a lack of high-quality data on microbiome changes that are associated with AD in humans.

While there are numerous studies claiming to identify AD-associated microbiome changes, there is little agreement between studies, emphasizing serious methodological challenges within the field. One of the most highly cited and well-respected studies on the human AD microbiome was published back in 2017, comparing the fecal microbiome of 25 control patients to that of 25 AD patients(Vogt, Kerby et al. 2017). The authors made efforts to account for a range of potential confounding variables, including age, sex, ethnicity, BMI, diabetes diagnosis, medication usage (though there were differences between groups), diet, and stool form, making it one of the most well-controlled studies published to date. Even almost a decade later—and despite a large uptick in the number of AD microbiome studies—these standards have rarely been met, let alone exceeded. Countless papers have been published without proper controls to account for dietary, lifestyle, and environmental factors that are known to influence microbiome composition. In addition, publications lack the proper sample size to account for the substantial variability that naturally occurs within the human microbiome. It is estimated that only roughly 10-20% of microbes are typically shared between two unrelated individuals, highlighting the incredibly high level of person-to-person variability in microbiome community structure(Seo and Holtzman 2024). When studies rely on sample sizes of

around 20-50 individuals as most currently published AD microbiome studies do, it is very difficult to overcome these levels of variability, and instead, results are likely to be heavily shaped by selection biases that skew the data based on other factors such as living environment, diet, or medication usage. In the past several years, researchers in other fields, such as the PD microbiome field, have finally begun to address some of these shortcomings, publishing papers with many hundreds of participants (Toh, Chong et al. 2022, Wallen, Demirkan et al. 2022). Similarly, a paper was recently published characterizing the microbiome composition of 519 participants with varied levels of cognitive impairment (n = 63-122 per group) (Jia, Ke et al. 2025). The authors also accounted for various potential confounding variables, including recent antibiotic usage and a number of co-morbidities (though notably no attempts to account for diet or medication usage were made), highlighting progress beginning to occur even in the AD field. As more rigorous studies begin to be performed, hopefully, more consistent trends will begin to appear between studies, providing clear targets for mechanistic studies. Once these targets have been identified, additional studies characterizing the effects of specific AD-associated microbes at the species and even strain level must be performed in order to determine whether individual bacteria may be modulating disease outcomes. In theory, this would allow for the identification of microbes that represent novel therapeutic targets.

4.3 The promise of microbes for personalized medicine

As our understanding of the specific effects of individual microbes continues to grow, one can imagine a time when microbes become a form of personalized medicine. The present study highlights how individual microbes have the capacity to shape neuroinflammatory tone in very unique ways that, in turn, modulate disease susceptibility. These microbes could therefore be used to adjust neuroinflammatory tone in a way that is beneficial for an individual's specific disease and even disease stage. For example, we demonstrate that *E. coli* enrichment inhibits microglia from entering the

"disease associated microglia" (DAM) activation state first characterized by Keren-Shaul et al. in 2017. Where in early stages of disease, DAM are thought to be protective, DAM are thought to be detremential later in disease progression, DAM are thought to be detrimental. Would it therefore be helpful to supplement with *E. coli* at later disease stages to inhibit the negative consequences of DAM? One could also imagine a wide range of contexts outside of AD where using microbes to modulate neuroinflammatory tone could be beneficial. Can we use bacteria such as *B. theta* or *C. celatum* that inhibit adaptive immune responses in the brain to reduce autoimmune diseases such as multiple sclerosis?

Admittedly, a lot more research is needed before bacteria-based personalized medicines are ready for clinical use. However, the present study can serve as a roadmap for future studies aimed at identifying microbiome-based therapeutic targets across disease conditions. Initial screening of potential disease-modulatory bacteria in wild-type animals provides insight into which bacteria may be beneficial or detrimental in specific disease contexts. The utilization of mono-colonized mice in this initial screen can be particularly beneficial in pinpointing the specific effects of each bacterium on the brain. Although the present work utilized bacterial type strains because human strain data was not available, ideally, initial screening would be done using human disease-relevant bacterial strains, as even strain-level differences have the potential to impact disease-relevant outcomes. While mono-colonization allows one to isolate the effects of a specific microbe on the brain, germ-free mice are known to display numerous developmental abnormalities, including widespread immune abnormalities and increased gastrointestinal and blood-brain barrier permeability that can confound study results(Cryan, O'Riordan et al. 2019). Therefore, complementing these studies with an enrichment-based approach in conventionally raised animals allows for the study of a bacteria's effects on the brain in a more naturalistic (albeit less controlled) environment. Finally, as done in the present work, candidate microbes identified during these initial studies must be tested in a model of disease.

Our results demonstrate that microbes can induce different activation states in wild-type animals compared to an AD mouse model, highlighting the need to study microbes in a disease-relevant context. To increase human relevance, ideally, bacteria would be tested in mouse models that most accurately represent the human condition. While the 5xFAD mouse model was used in the present work, Appendix A outlines some of the limitations of these overt overexpression-based AD models and highlights a set of newer APP knock-in models that may more accurately recapitulate the human condition(Blackmer-Raynolds, Lipson et al. 2025). The selection of the most relevant mouse model for each disease of interest is essential for the effective translation of pre-clinical data into clinical populations.

I strongly believe that this thorough multi-step preclinical evaluation of targeted disease-relevant microbes will allow for the identification of novel microbiome-based treatment targets across disease contexts. However, I would caution against the premature, wide-sweeping use of probiotic treatments in patients without proper preclinical studies for the very same reasons I see such promise for personalized medicine. Where I am suggesting a very nuanced view of bacteria, highlighting contexts where the same microbe can be beneficial or detrimental, the current definition of "probiotics" makes wide-sweeping assumptions that "good bacteria" are beneficial in all contexts. Just as no medication is helpful in treating all diseases, no bacteria will improve symptoms in all conditions. In fact, there is increasing evidence to suggest that just the opposite may be true, with traditional probiotic species such as a number of *Lactobacillus* and *Bifidobacteria* species being elevated in patients with Parkinson's disease (Romano, Savva et al. 2021, Wallen, Demirkan et al. 2022). As a research community, it is therefore essential that we recognize both the incredible potential of microbiome-based therapeutics, but also the nuance and dangers of oversimplification. The microbiome has the capacity to influence every single cell type in the brain, allowing for targeted treatment across neurological conditions, however, care must be taken to ensure that species- and strain-specific effects

of bacteria on the brain are taken into account and each bacterial type is used in the proper clinical context.

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Appendix 1: Longitudinal characterization reveals behavioral impairments in aged APP Knock in mouse models

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A1.1 Abstract

APP knock-in (KI) mice serve as an exciting new model system to understand amyloid beta (Aβ) pathology, overcoming many of the limitations of previous overexpression-based model systems. The APP^{SAA} mouse model (containing the humanized APP with three familial Alzheimer's disease mutations) and the APP^{WT} control (containing wildtype humanized APP) are the first commercially available APP KI mice within the United States. While APP^{SAA} mice have been shown to develop progressive Aβ pathology and neuroinflammation, the age at which behavioral and cognitive impairments begin to develop has yet to be described. Therefore, we performed an in-depth longitudinal study over 16 months, assessing cognition in these two strains, as well as assessments of motor function. While no cognitive deficits are observed in either genotype throughout the first year of life, 16-month-old APP^{SAA}, but not APP^{WT} mice show initial signs of spatial memory decline. In addition, both genotypes display impaired motor function at the same age. Together, this data identifies the age-dependent tipping point where behavioral deficits appear, providing an essential foundation for future studies using these model systems.

A1.2 Introduction

Establishing and characterizing animal models that accurately recapitulate pathological and behavioral outcomes of human disease remains an essential component—and limiting factor—to understanding disease etiology and identifying novel treatment targets. Rodent models of amyloid beta (Aβ) pathology—a key hallmark of Alzheimer's disease (AD), as well as a prominent co-pathology in Parkinson's disease (PD), Lewy body dementia (LBD), and other amyloid diseases—have been utilized for decades (Gotz, Bodea et al. 2018, Myers and McGonigle 2019). These models have provided important insights into the role of Aβ in neurodegeneration and other physiological processes. Early rodent models of Aβ pathology were first developed by overexpressing the human

amyloid precursor protein (APP) gene containing highly amyloidogenic mutations associated with familial AD (FAD) in humans (Games, Adams et al. 1995, Hsiao, Chapman et al. 1996, Sturchler-Pierrat, Abramowski et al. 1997). The majority of amyloid transgenic mouse models in use today follow a similar, overt overexpression approach, driving high expression of various combinations of transgenes of APP, presenilin, tau, and alpha-synuclein (Chesselet 2008, Gotz, Bodea et al. 2018). However, there is increasing recognition that these overexpression approaches also have a number of important limitations. These include random gene integration, ectopic expression, overrepresentation of specific splice variants, non-physiological drivers of protein expression, and an inability to assess transcriptional regulatory impacts on the gene of interest, all of which confound experimental results (Jankowsky and Zheng 2017).

In response to these limitations, various knock-in (KI) models of amyloid pathology have been created in which the murine gene of interest is knocked-out and replaced with a humanized transgene either at the locus itself or in *trans* (Kuo, Li et al. 2010, Janezic, Threlfell et al. 2013, Saito, Matsuba et al. 2014, Gotz, Bodea et al. 2018, Saito, Mihira et al. 2019, Sakakibara, Sekiya et al. 2019, Serneels, T'Syen et al. 2020, Baglietto-Vargas, Forner et al. 2021, Xia, Lianoglou et al. 2022). These KI models overcome many of the limitations of overexpression-based models because the transgenes are expressed closer to physiological levels and are driven by native promotors, generating much excitement within the research community (Jankowsky and Zheng 2017). The first commercially available KI model of Aβ pathology in the United States, the APP^{SAA} mouse, carries humanized APP with three mutations associated with FAD (the Swedish, Arctic, and Austrian mutations) (Xia, Lianoglou et al. 2022). The APP^{SAA} mouse develops amyloid and tau pathology, neurodegeneration, neuroinflammation, and neurovascular deficits that recapitulates elements of human disease (Xia, Lianoglou et al. 2022, Whittaker, Akhmetova et al. 2023, Kim, Cruz et al. 2024, Lu, Shue et al. 2025). In addition, studies have demonstrated hyperactivity behavior at 18 months

(Xia, Lianoglou et al. 2022), cognitive impairments on contextual and cued fear conditioning tests at 12-13 months (Lu, Shue et al. 2025) and object recognition and radial arm maze deficits at 7.5 months (Whittaker, Akhmetova et al. 2023). However, it is currently unknown when these behavioral changes begin to develop and how they progress as pathology continues to increase. To address this, here we performed an in-depth, longitudinal characterization of various aspects of rodent behavior known to be impacted by amyloid pathology including anxiety-like behaviors, learning and memory, as well as motor function in APP^{SAA} mice. These behavioral assessments were preformed side-by-side in APP^{WT} mice, another under-characterized APP KI model. APP^{WT} mice encode humanized, wild-type APP without any FAD mutations and have significantly less Aß accumulation than APP^{SAA} mice(Lu, Shue et al. 2025). We initially predicted that both mouse strains would display progressive behavioral impairments due to the presence of the human APP gene, with the APP^{SAA} mice displaying exacerbated impairments compared to mice harboring the wildtype human gene. While we observed human Aβ accumulation in the brains of both genotypes, which was significantly higher in the APP^{SAA} mice, neither strain developed progressive cognitive or motor deficits within the first year of life. However, APP^{SAA} mice were found to display significant learning and memory deficits on the object location and Barnes maze tests at 16 months of age. In addition, both APP KI genotypes displayed motor impairments beginning at 16 months. This age therefore represents the tipping point whereby APP KI mice display age- and genotype-dependent cognitive impairment, an essential parameter for the study of disease modifying factors of amyloid pathology.

A1.3 Materials and methods

Animal husbandry. Female and male APP^{SAA} KI (B6.Cg-Apptm1.1Dnli/J Strain #:034711) and APP^{WT} KI (B6.Cg-Appem1Adiuj/J Strain #:033013) mice were acquired from Jackson Labs through a kind gift by Dr. Srikant Rangaraju (Emory University) and maintained as homozygotes. The APP^{SAA} model was created by humanizing the Aβ region of the APP gene using R684H, F681Y, and

G676R mutations, then adding 3 additional FAD mutations: the KM670/671NL (Swedish) mutation in exon 16 as well as the E693G (Arctic) and T714I (Austrian) mutations in exon 17 (Xia, Lianoglou et al. 2022). The APP^{WT} mouse contains a humanized Aß1-42 region (G601R, F606Y, R609H in the mouse gene, corresponding to amino acid positions 676, 681, 684 in the human *APP* locus), but no additional FAD mutations. Both genotypes were maintained on a C57BL/6J background. Mice were genotyped by PCR using primers and conditions per the vendor (Jackson Labs). Mice were housed with 2-5 sex and age matched cage mates of the same genotype within unenriched microisolator cages in a central, specific pathogen-free vivarium on ventilated racks, with food (LabDiet: 5001) and water provided *ad libitum* and a 12:12hr light-dark cycle. All animal experiments were performed during the animal's light cycle. Animal husbandry and experiments were performed in accordance with AVMA guidelines and approved by the Institutional Animal Care and Use Committee of Emory University (PROTO201900056).

Overview of Cognitive Behavioral Testing. Since, APP^{SAA} mice have been shown to develop pathology starting at 4 months of age(Xia, Lianoglou et al. 2022) we began longitudinal cognitive testing monthly at 4, 5, and 6 months of age to see if early pathology would impact behavior, however, when no cognitive impairments were observed the duration of testing was spread out and repeated again on the same animals at 12 months, when previous reports(Lu, Shue et al. 2025) had shown deficits. Finally, to test an even later age and mitigate any potential confounds of repeat testing, mice were also tested cross sectionally at 2-3 months old and 16 months old. Each genotype and age included 9-13 mice with roughly equal numbers of males and females as indicated in the associated data file. At each timepoint, the following tests were performed in order: open field test, object location test, Y maze, and Barnes maze. Before the start of any test, mice were habituated to the testing room in their home cage for 1hr. All behavioral tracking and analysis was performed using EthoVision XT software (Noldus Information Technology, Wageningen, the Netherlands)

and the testing arenas/objects were cleaned between trials with 70% ethanol to eliminate olfactory cues.

Open Field Test (OFT): During the OFT, mice were placed in a 45cm square open field box for 10 minutes while distance traveled and time spent in the center was recorded as measures of motor and anxiety-like behavior. *Object location test* (OLT): 24 hours post OFT, the OLT was run as described step-by-step in (Blackmer-Raynolds, Krout et al. 2024) to assess short-term spatial memory. Briefly, mice were placed in an open field with landmarks on 3 out of the four walls to allow for spatial orientation. During the initial study phase, mice were placed in the box with two identical copies of an object and allowed to explore freely for 10 minutes. The mice underwent a 10-minute retention delay in their home cage before being returned to the field for 5 minutes, with one of the two objects placed in a new location. Object exploration was considered time spent with the mouse's nose within 2cm of an object. Exploration ratio = moved object exploration/total object exploration. An exploration ratio significantly above chance levels of 0.5 is indicative of intact memory as mice generally seek out the moved object. *Y-maze:* Mice were then tested on the Y-maze to evaluate spatial working memory, as described in detail in (Blackmer-Raynolds, Krout et al. 2024). Mice were allowed to freely explore a Y shaped maze for 8 minutes while entries into each arm were recorded. Percent alternation = total alternations (consecutive entries into 3 arms before repeating any arms) / maximum possible alternations (total entries minus 2). Mice with intact working memory display higher percent alternation as mice prefer to explore previously unvisited areas. *Barnes Maze:* Barnes maze testing adapted from (Attar, Liu et al. 2013), and described in detailed in (Blackmer-Raynolds, Krout et al. 2024) was used to assess spatial learning and memory. Testing occurred on a 92cm diameter, 20 hole, Barnes maze (MazeEngineers) over a 6-day period, with one habituation trial, five 3-minute training trials, and a 72-hour probe trial. During habituation, mice were placed on the maze with bright lights and white noise (66-70 dB) playing for 20 seconds before

being gently guided to an escape hole leading to a dark box. Upon entry to the box, the white noise was turned off and the mice were allowed to rest in the escape box for 2 minutes. During each training trial, mice were once again placed on the maze with white noise playing and allowed to explore for up to 3 minutes. Upon entry into the escape box, the noise was turned off and mice were allowed to rest for 1 minute. Mice that did not enter the escape box after 3 minutes were guided to the proper hole. 72 hours after the final training trial, mice were returned to the maze with the escape box removed and their behavior was observed for a 90 second probe trial. Primary latency (time until the mouse first checked the escape hole) was recorded for each trial. During repeat testing, the location of the escape hole was shifted 90 degrees to prevent mice from remembering the previous location of the escape hole. The extra-maze cues remained the same throughout all testing sessions.

Motor behavior assessments. Since motor impairments typically develop later in the progression of amyloid pathology (O'Leary, Mantolino et al. 2020), motor function testing was performed cross sectionally at 3, 12, and 16 months of age as follows. *Adhesive removal:* The adhesive removal test was performed as described in (Krout and Sampson 2024) , by placing an adhesive sticker onto the mouse's nose and quantifying the time needed to remove the sticker across three consecutive trials. This test provides insight into both sensory perception and fine motor skills. *Wirehang:* The wirehang test was performed as described in (Sampson, Krout et al. 2024) to evaluate muscle strength and function. Briefly, mice were placed on a 43cm square wire screen (made up of 12 mm squares of 1 mm diameter wire), turned upside down, and the time the mouse held onto the screen was recorded and averaged across three consecutive trials. *Hindlimb rigidity:* Hindlimb scoring was performed on a scale of 0-3 based on extent of hindlimb clasping adapted from (Lieu, Chinta et al. 2013) and described in (Sampson and Krout 2024) on two separate days by two independent scorers. Greater hindlimb clasping is seen in mice with motor deficits and is indicative of neurodegenerative disease

progression.

Tissue Collection and Aβ Quantification. At 12 months of age, mice were humanely euthanized under isoflurane anesthesia and perfused with PBS. Aß levels were quantified in the hippocampus since this is a region known to have the highest Aß burden in APP^{SAA} mice (Lu, Shue et al. 2025). Hippocampal tissue was dissected, flash frozen, and protein extracted using a two-part fractionation protocol (Blackmer-Raynolds and Sampson 2024). Tris soluble (1M Tris HCL, 0.5M MgCl2 and 0.1M EDTA- pH 7.8) and Triton soluble (1% Triton-X100) fractions were run on the Meso Scale Discovery V-PLEX human Aβ peptide kit (K15200E) according to the manufacture's guidelines. The resulting protein values were normalized to frozen tissue weight (rather than total protein concentration) since the total protein volume of the less soluble fractions may be influenced by the amount of insoluble Aß present in the tissue.

Statistical Analysis. As indicated in the figure legends, data are expressed as mean ± SEM. Statistical tests were performed using GraphPad Prism 8. Longitudinal data was analyzed using repeated measures one- or two-way ANOVAs and cross-sectional data was measured using standard ANOVAs. The OLT outcome was measured using a one sample t test compared to 0.5 chance level, and Barnes maze training trials were analyzed by comparing the area under the curve (AUC) from all five training trials. All raw numerical data are included with the manuscript as a supplemental file.

A1.4 Results

Limited cognitive impairment is observed longitudinally through twelve months of age. In order to test the hallmark, progressive, cognitive impairments observed in amyloid diseases, and other amyloid mouse models, we examined both APP KI genotypes across a battery of behavioral tests through aging. Mice were first evaluated longitudinally starting at 4 months of age (when pathology begins to develop in the APP^{SAA} mice (Xia, Lianoglou et al. 2022, Kim, Cruz et al. 2024)) to assess whether cognitive decline develops within the first year of life. However, throughout the

first 12 months of age, neither genotype displayed progressive cognitive impairment on any of the behavioral tests (Fig. A1.1). In the object location test (OLT), the APP^{SAA} mice maintained a consistent discrimination of the object in a novel location—represented by an exploration ratio significantly above 0.5 chance levels (Fig. A1.1 A). However, the APP^{WT} genotype consistently performed no better than chance, with a trend towards novelty preference ($p = 0.053$) only appearing at 12 months (Fig. A1.1 B). This is despite no significant difference in total object exploration between the two genotypes at any timepoint, but a significant overall decline in object exploration with repeat testing in the APP^{WT} mice at 12 months (Fig. A1.2 A).

Similarly, in the Y-maze test of spatial working memory, we observed no progressive loss of working memory (indicated by a decline in percent alternation) in either genotype through 12 months of age (Fig. A1.1 C, D). Of note, however, there was a significant negative correlation between percent alternation and the total number of arm entries for both genotypes, especially the APP^{SAA} mice, suggesting the animal's activity level in this specific test may confound an interpretation of their cognitive performance (Fig. A1.2 B-C). To determine general locomotor activity and evaluate potential anxiety-like behaviors that could impact cognitive outcomes, the open field test (OFT) was also performed. While we observed a gradual decrease in distance traveled over time—a common finding as animals become habituated to the repetitive testing environment(Bolivar, Caldarone et al. 2000)—differences in distance traveled between the two genotypes only appeared at 4 months of age (Fig. A1.2 D). In addition, 4-month-old APP^{SAA} mice spent more time in the center during the open field compared to APP^{WT} at the same age suggesting potential genotype-dependent effects in anxiety-like behavior that dissipates with age (Fig. A1.2 E).

In the Barnes maze, both genotypes were able to quickly learn the location of the goal box even at 12 months of age (Fig. A1.1 E, F). In fact, mice in both genotypes showed a median primary latency (time to first identify the target hole) below 30 seconds (as is typically seen after successful

Barnes maze training (Attar, Liu et al. 2013)) within the first two training trials, suggesting that the mice learned the location of the goal box during habituation and the first training trail. While primary latency did not significantly decrease over subsequent training sessions, this is likely due to a plateau effect rather than a learning deficit. While repeat testing could theoretically decrease initial primary latency measures if mice remember the location of the escape hole from the previous test, no significant difference in primary latency was observed during any training trial, suggesting that the tests were far enough apart to not require reversal learning (Fig. A1.1 E, F). Following a 72hr retention interval, all ages and genotypes showed similar or improved primary latency, suggesting no progressive loss of memory over time (Fig. A1.1 G, H).

To confirm the presence of increased human amyloid beta (Aβ) in each KI model, we performed detergent fractionation of hippocampal tissue lysates at 12 months of age. In line with prior reports(Xia, Lianoglou et al. 2022, Lu, Shue et al. 2025), we observed the presence of both Tris soluble and detergent soluble Aβ by ELISA, with generally increased levels in the APP^{SAA} genotype (Fig. A1.1 I, J). Given the behavioral outcomes, we conclude that the presence of amyloid burden at this age is not sufficient to induce cognitive impairment in these hallmark behaviors.

APP^{SAA} mice display cognitive impairment at 16 months of age. While we did not observe cognitive impairment through 12 months of age, similar models can begin behavioral deficits later in life (Saito, Matsuba et al. 2014). We therefore performed a cross-sectional analysis between independent cohorts of young, 2–3-month-old APP^{WT} and APP^{SAA} mice compared to individuals aged through 16 months-old (Fig. A1.3). While 2-3 month APP^{SAA} mice showed a clear trend towards a novelty preference on the OLT ($p = 0.054$), 16 month APP^{SAA} mice had a novelty preference score that was no different than chance levels (despite spending similar time exploring the objects, Fig. A1.4 A), suggesting that they were no longer able to remember which object had been moved (Fig. A1.3 A). There was, however, still no significant difference in percent alternation

on the Y maze based on age or genotype (and no correlation between percent alternation and number of entries, Fig. A1.4 B-C), suggesting that all mice have similar working memory (Fig. A1.3 B). Finally, in the Barnes maze, 16-month APP^{SAA} mice showed a significant learning and memory deficit. Compared to both young APP^{SAA} and 16 month APP^{WT} mice, 16 month APP^{SAA} mice never were able to learn the escape hole location, with a significantly higher training AUC than the young mice of either genotype (Fig. A1.3 C,D). Similarly, 16-month APP^{SAA} mice took significantly longer to find the escape hole during the probe trial than the other groups (Fig A1.3 E). While the mixed-sex cohorts used in the present study are not sufficiently powered to evaluate sex differences, 2-3 month male APP^{SAA}, appear to have worsened cognitive impairment compared to females, highlighting the need for future studies to investigate sex differences in these models (Fig A1.4 D). Together, these results suggest that APP^{SAA} but not APP^{WT} mice display cognitive deficits at 16 months old.

Since motor impairments are associated with the development of amyloid pathology, motor testing was also performed within both APP KI genotypes. Mice were tested cross-sectionally, at 3, 12, and 16 months. While both genotypes showed intact sensorimotor function during the nasal adhesive removal test and motor function on the wire hang test and at 3 and 12 months, 16-month-old mice of both genotypes show significant impairments in these behaviors (Fig. A1.4 E-F). In addition, APP^{SAA} mice fell from the wirehang test more quickly than the APP^{WT} mice at 16 months, suggesting a greater motor impairment in this genotype (Fig. A1.4 F). Even at 16 months neither genotype displayed limb rigidity on the hind limb test (Fig. A1.4 G). APP^{SAA} mice were heavier at 16-months of age than APP^{WT} animals (Fig. A1.4 H-I), which may contribute to the poorer performance in the wirehang test. However, both animals were impaired in this test irrespective of body weight and body weight would not be expected to contribute to the impaired adhesive removal test. In the OFT, APP^{WT} mice traveled further than the APP^{SAA} mice, particularly at 3-month of age

(Fig. A1.4 J). In addition, there was a significant reduction in the total distance traveled between the young 2–3-month APP^{WT} mice and the 16-month APP^{WT} mice (Fig. A.14 J). There was, however, no difference in time spent in center across genotypes or ages, suggesting that anxiety-like behavior remains stable up to 16 months (Fig. A.14 K).

A1.5 Discussion

KI models, such as the APP-KI strains used in this study, represent an important tool to understand etiological mechanisms that underlie amyloid pathologies and therapeutic interventions aimed at their clearance. By removing the presence of the murine amyloid ortholog, potential confounding interactions between the human and murine amyloids are avoided. Transcriptional control by the native human promoter in these models further allows a clearer understanding of how amyloid proteins respond to disease-relevant insults, such as immune modulation, metabolic input, or environmental exposures. In order to effectively use these models, however, it is critical to evaluate baseline pathologies and behaviors to have a foundation to explore such perturbations.

The two APP KI genotypes used in this study are emerging and recently described model systems that currently lack complete characterization. While prior studies have characterized amyloid pathology and neuroinflammation (Xia, Lianoglou et al. 2022, Kim, Cruz et al. 2024) and demonstrated behavioral abnormalities late in life in the APP^{SAA} mice (Xia, Lianoglou et al. 2022, Whittaker, Akhmetova et al. 2023, Lu, Shue et al. 2025), to our knowledge, there is no published dataset on the age-related development and progression of cognitive behaviors of either KI genotype. We therefore set out to identify a behavioral "tipping point," an age where these genotypes began to show cognitive impairment or an age where the more pathogenic APP^{SAA} genotype separated behaviorally from the APP^{WT} genotype. Such a timepoint is important for timing potential interventions that seek to accelerate or diminish disease outcomes, directly test etiological contributions, or evaluate therapeutic interventions.

APP^{SAA} mice have been reported to display progressive amyloid pathology and neuroinflammation starting at 4 months of age (Xia, Lianoglou et al. 2022, Kim, Cruz et al. 2024). We therefore hypothesized that these mice would display cognitive impairment as pathology progressed. However, despite assessing behaviors longitudinally for 12 months and confirming the presence of human Aβ accumulation in both mouse models, we were unable to detect progressive cognitive impairment in either genotype within the first year. This stands in contrast to previously published papers showing deficits on the radial arm maze and object recognition test in APP^{SAA} mice at 7.5 months(Whittaker, Akhmetova et al. 2023) and in cued fear conditioning in APP^{SAA} and contextual fear conditioning tests in both genotypes at 12-13.5 months old compared to WT C57BL/6J controls (Lu, Shue et al. 2025). This discrepancy is likely due to differences in the cognitive tests performed and overall experimental design. In the present study, our main goal was to identify the age at which cognitive decline develops in each genotype, so comparisons were made within the same mice at various timepoints rather than across genotypes. This allows us to identify an age-related tipping point within a single genotype/animal but may be less sensitive to subtle genotype dependent differences in cognitive performance compared to WT animals. In addition, using a repeated measures design may mask subtle cognitive deficits, as mice may have an easier time with a task that they have performed before. However, even at the youngest ages and within the APP^{WT} genotype where no cognitive impairment is expected, repeat testing did not result in significantly improved performance on any of the cognitive tests. In fact, the only cognitive test that showed improvement was the 12 month APP^{SAA} Barnes maze probe trial, further emphasizing that these mice have intact cognition at this age.

While we were unable to detect significant age-related cognitive decline in the first year of life, each genotype displayed significant behavioral impairments at 16 months of age. Both genotypes showed significant motor deficits on the wire hang and adhesive removal test, suggesting

that the human APP KI is sufficient to modulate motor outcomes at this age, regardless of the presence of FAD mutations. In addition, 16-month-old APP^{SAA}, but not APP^{WT} mice displayed spatial memory and learning deficits on the OLT and Barnes maze tests. Of note, however, all of these tests were performed cross sectionally rather than longitudinally, so it is difficult to directly compare these results to those of previous ages. Nevertheless, these results suggest that 16 months likely represents the age at which age- and genotype-dependent behavioral deficits associated with amyloid disease manifest in these mice. While a subtle exacerbation of cognitive impairment on the Barnes maze is observed in 2-3 month male APP^{SAA} compared to females, the mixed-sex cohorts used in the present study were not sufficiently powered to reasonably detect sex differences. This stands in contrast to a recently published study showing contextual and cued fear conditioning deficits in female, but not male mice at 12-13.5 months of age (Lu, Shue et al. 2025), highlighting the need for future studies to further evaluate sex differences in these models.

Of note, the APP^{WT} mice performed at chance levels on the OLT at all timepoints, suggesting possible behavioral abnormalities even in young mice. While the APP^{WT} mouse is considered a control for the APP^{SAA} genotype, they are themselves a KI model and therefore may display their own behavioral deficits caused by the insertion of the wildtype humanized APP gene. However, lack of observable cognitive deficits on the other cognitive tests, suggests that this deficit is either very specific to the type of short-term object location memory tested, or is due to a confounding variable such as differences in motivation, hyperactivity, or visual acuity. Indeed, 2–3-month-old APP^{WT} mice display increased distance traveled on the OFT compared to APP^{SAA} mice. While this trend remains at 16 months of age, there is a significant decrease in distance traveled in the APP^{WT} mice with age, perhaps explaining why the older APP^{WT} mice have OLT discrimination ratios that are almost significantly above chance levels. Together, these results suggest that OLT may not be a suitable test for evaluating cognitive decline in APP^{WT} mice. Use of cognitive test that are

not shaped by hyperactivity would allow for a more accurate evaluation of age-related cognitive performance in this model.

We appreciate that tests of murine cognitive function have a number of caveats, including environmental variability, as well as potential confounding effects of anxiety-like behavior and motor attributes. Nonetheless, the behavioral tests utilized in this study are well-evaluated to identify cognitive impairments across a range of AD models (Webster, Bachstetter et al. 2014). When used in combination with measures of anxiety-like behavior and motor functions, these tests provide important insights into age-related cognitive impairment within these models that can be qualitatively compared to other more established mouse models. Within established APP KI models, there is considerable variability in the age of cognitive decline depending on the presence of specific FAD mutations. For example, the $APP^{NL\text{-}G\text{-}F}$ model (containing the Swedish, Iberian, and Artic mutations) has been shown to display cognitive impairment on the Y maze starting at 6 months of age, but the $APP^{NL\text{-}F}$ model (containing only the Swedish and Iberian mutations) does not show impairment in this test until 18 months (Saito, Matsuba et al. 2014). While the APP^{SAA} model contains the Swedish and Artic mutations like the $APP^{NL\text{-}G\text{-}F}$ model, the presence of the Austrian mutation, rather than the Iberian mutation, may impact the age of onset for cognitive decline, which we observe at 16 months of age. While cognitive decline was not observed in APP^{WT} mice at 16 months of age, it is possible that cognitive impairment could appear in this model at even later ages or with more sensitive tests. For example, contextual fear conditioning deficits have been reported in 12-13.5 month old APP^{WT} mice (Lu, Shue et al. 2025). Wildtype C57BL/6J mice are reported to display cognitive and motor impairments at 18-20 months of age, so it is possible that the APP^{WT} mice will not begin to show more striking cognitive deficits in other behavioral paradigms until this age (Benice, Rizk et al. 2006, Barreto, Huang et al. 2010, Justice, Carter et al. 2014).

No single AD mouse model can fully recapitulate the human condition; however, when

properly characterized, model systems are essential tools to address specific biological and translational questions. The present study provides a longitudinal characterization of the behavioral phenotypes of APP^{SAA} and APP^{WT} mice in a mixed-sex cohort over the course of 16 months, providing foundational behavioral data to inform future work. We have identified an age at which APP^{SAA} mice show significant cognitive decline compared to APP^{WT} mice, providing a critical behavioral window for studies designed to limit or exacerbate behavioral defects. Further, we have identified age-related motor dysfunctions in both mouse models that will be important to consider both in the interpretation of cognitive behaviors and in the study of co-morbidities of amyloid diseases. Future studies into how various insults shape pathological and behavioral outcomes in these relevant Aβ-dependent mouse models will provide substantial insights into how various factors interact to promote disease.

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A.1.7 Figures

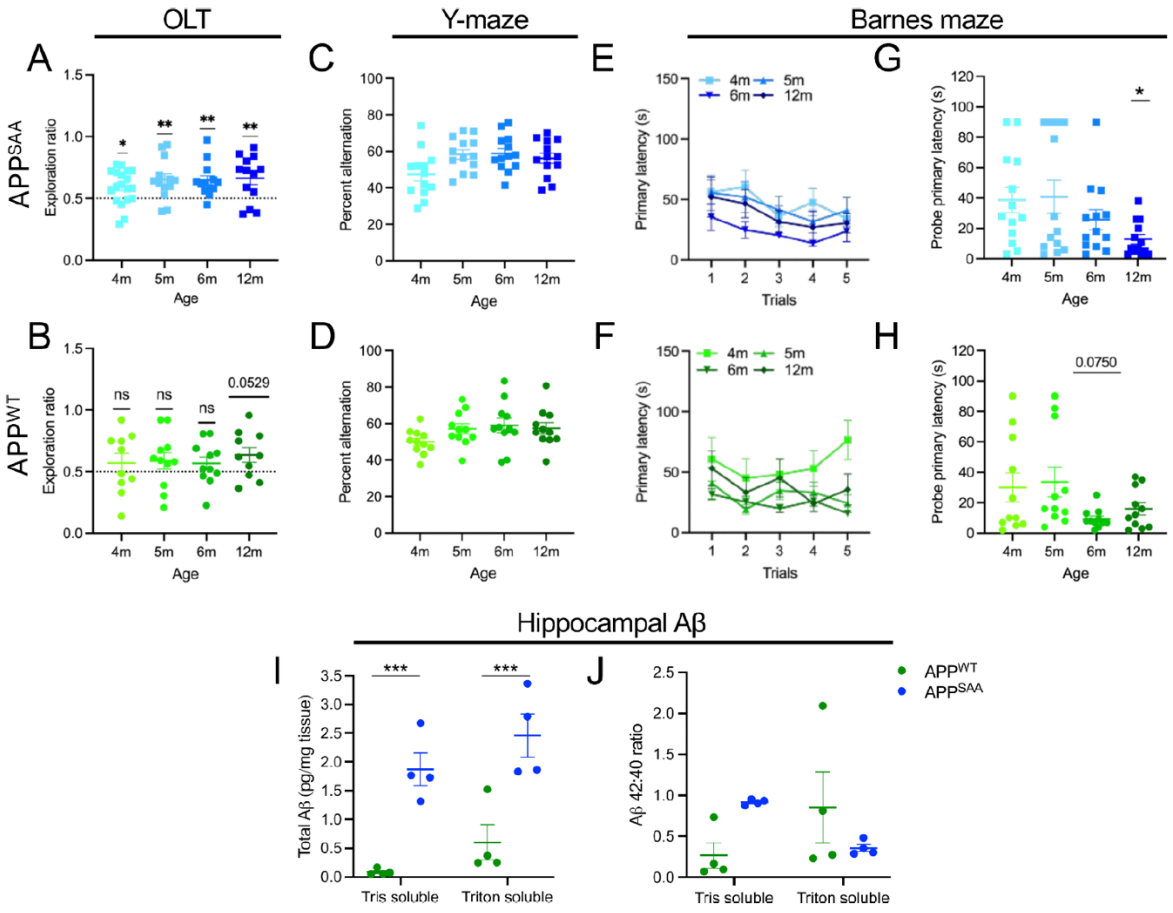

Figure A1.1. **APP-KI mice do not show progressive cognitive impairment through 12 months of age.** Male and female APP^{SAA} (**A,C,E,G**) and APP^{WT} (**B,D,F,H**) mice were tested longitudinally from 4 through 12 month (m) of age, at the indicated time points across a battery of cognitive behavior tests. (**A,B**) Exploration ratio in the object location test (OLT). (**C,D**) Percent alternation in the Y-maze. (**E,F**) Barnes maze performance during longitudinal training period and (**G,H**) 72 h probe trial. (**I,J**) Tris soluble and Triton soluble hippocampal Aß analyzed by multiplex ELISA. (**I**) Total Aβ including Aβ38, Aβ40, and Aβ42 (**J**) Aβ 42:40 ratio. A significant genotype by solubility interaction effect was observed in Aβ 42:40 ratio (p=0.0297). Points represent individuals (excluding (**e,f**) where points represent the group mean), bars represent the mean±SEM. n=10–11 APP^{WT} and 13 APP^{SAA} for (**A-h**) and n=4 for (**I-J**). Data analyzed by one sample t test compared to 0.5 chance level for (**A-B**) repeated measures ANOVA and Dunnett's multiple comparisons test to compare each age to 4 m timepoint (**C-H**) and 2-way ANOVA with Fisher's LSD post-hoc tests (**I-J**). *p≤0.05; **p≤0.01; ***p≤0.001.

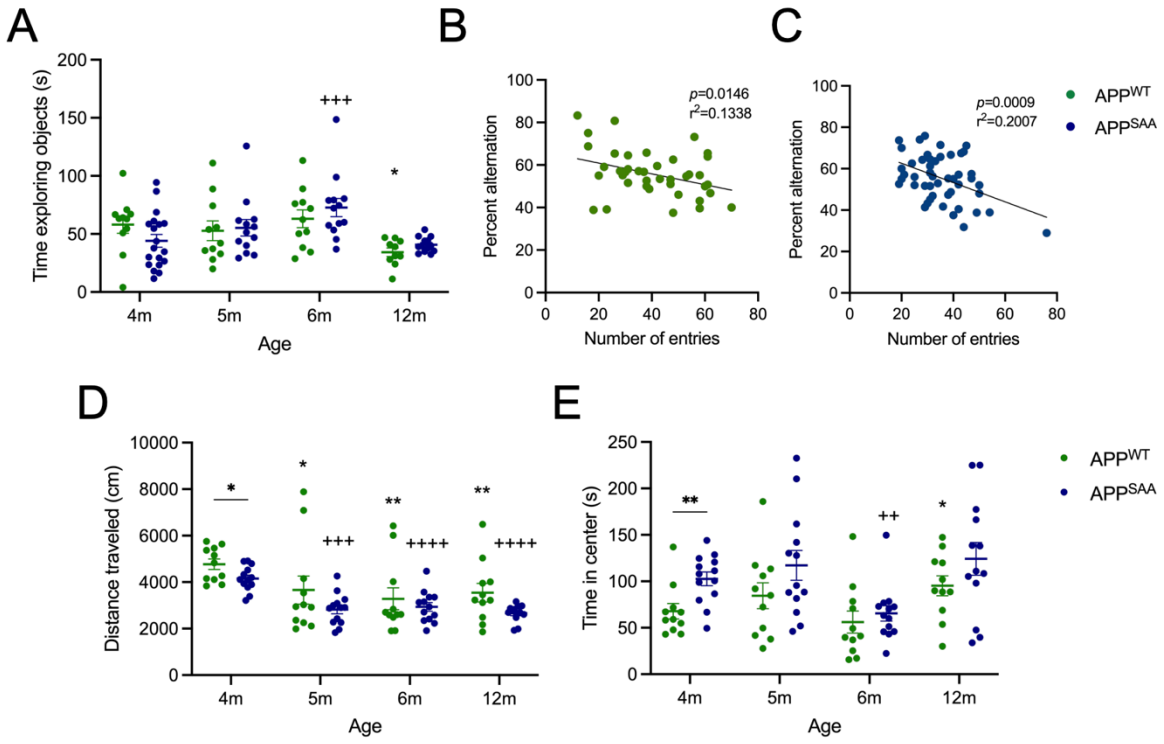

Figure A1.2. **Limited behavioral abnormalities appear in APP-KI mice through 12 months of age. A**) Total object exploration during OLT. **B, C**) Correlation between number of entries and percent alternation during the Y maze for APP^{WT} (**B**) and APP^{SAA} (**C**) mice respectively. **D, E**) Male and female $APPS^{AA}$ and APP^{WT} mice were tested longitudinally on the open field test from 4-12 months (m) of age. **D**) Total distance traveled and **E**) time spent in center during 10-minute testing period. Points represent individuals, bars represent the mean and SEM. n= 10-11 APP^{WT} and 13 APP^{SAA} for **A**, **D**, & **E**. Data analyzed with a 2-way ANOVA with Fisher's LSD post hoc test for **A**, **D**, & **E** and simple linear regression for **B**-**C**. *p≤0.05; **p≤0.01; ***p≤0.001 compared to 4m APP^{WT} mice. +p≤0.05; ++p≤0.01;+++p≤0.001, ++++p≤0.0001 compared to 4m APP^{SAA}.

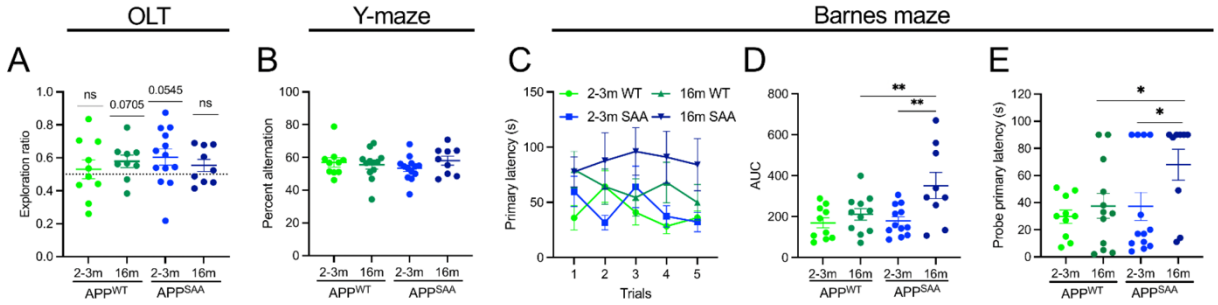

Figure A1.3. **APP^{SAA} but not APP^{WT} mice show spatial learning and memory deficits at 16 months of age.** Young (2–3-month-old) and 16-month-old male and female APP^{SAA} and APP^{WT} mice were tested cross sectionally on a range of cognitive tests. (**A**) Exploration ratio in the object location test (OLT). (**B**) Percent alternation in the Y-maze. (**C-E**) Barnes maze performance during longitudinal training period showing both (**C**) primary latency and (**D**) area under the curve (AUC). (**E**) 72 h probe trial primary latency. Points represent individuals (excluding **C**, where points represent the group mean), bars represent the mean and SEM. n=9–12 APP^{WT} and 9–13 APP^{SAA}. Data analyzed by one sample t test compared to 0.5 chance level for (**A**), 2-way ANOVA with Fisher's LSD post hoc test for (**B,D** and **E**). *p≤0.05; **p≤0.01.

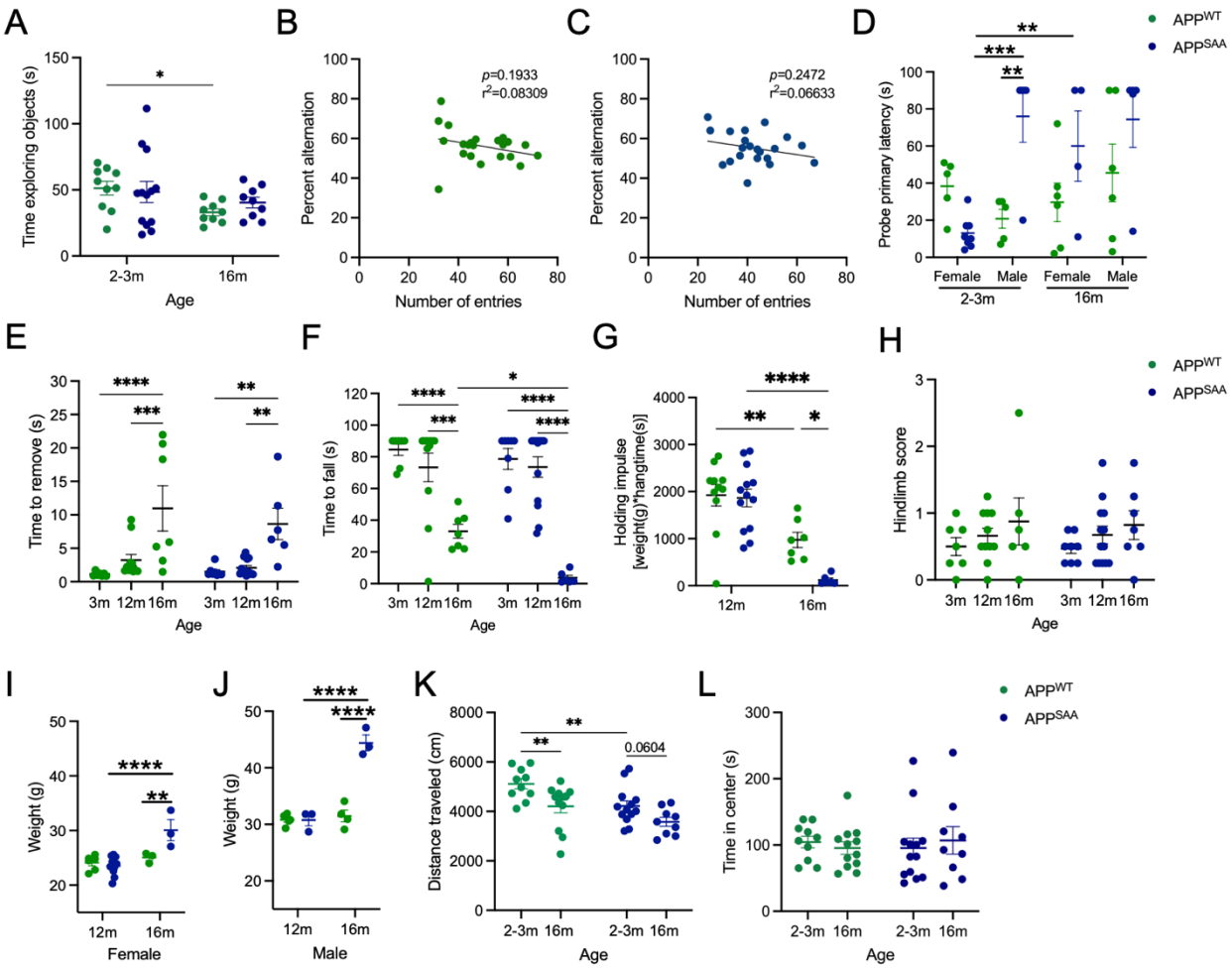

Figure A1.4. APP-KI mice show age related motor impairments regardless of genotype. Male and female APP^{SAA} and APP^{WT} mice were tested cross sectionally at 2-3, 12, and 16 months (m) of age. **A**) Total object exploration during OLT. **B,C**) Correlation between number of entries and percent alternation during the Y maze for APP^{WT} (**B**) and APP^{SAA} (**C**). **D**) Primary latency in the Barnes maze, from Figure A1.3E, separated by biological sex. **E**) Time to remove a nasal adhesive in sticker removal test. **F**) Time to fall on the wire hang test. **G**) Hanging Impulse score. **H**) Hindlimb rigidity score. **I, J**) Body weight of female (**I**) and male (**J**) mice at 12 and 16m of age. **K**) Distance traveled and **L**) time spent in center during open field test. Points represent individuals, bars represent the mean and SEM. n= 7-11 APP^{WT} and 8-13 APP^{SAA}. Data analyzed by 2-way ANOVA with Fisher's LSD post-hoc tests comparing each genotype and age (or sex) for **A, D-K** and simple linear regression for **B-C**. *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001.

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