

Diverse mouse models reveal genetic context controls
the molecular and neural landscape of the aged and
Alzheimer's disease brain.

A thesis submitted by

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Abstract

The predominance of age-related diseases such as Alzheimer's disease (AD) are projected to double over the next several decades, which is attributed to the rapidly growing aging population. Since only a handful of therapies have been approved that mildly improve AD dementia symptoms, understanding the underlying cellular and molecular mechanisms that drive cognitive decline is of the utmost importance. AD is heterogeneous, governed by a host of factors including variation in an individual's genetic background. Recent work suggests genetic variants are critical to defining susceptibility and resilience to AD cognitive symptoms, but current mouse models lack the complexity to evaluate this. In this thesis I leveraged multiple genetically diverse mouse models to identify and evaluate the molecular and cellular processes that drive susceptibility and resilience to AD, as well as resistance to aging. I first compiled multidimensional data from the genetically diverse AD-BXD mouse panel and performed statistical modeling to identify gene co-expression networks that were the most predictive of individual differences in genetically-driven cognitive outcomes (**Chapter 2**). I then leveraged previous studies that identified the laboratory inbred C57BL/6J (B6) mouse strain as susceptible and wild-derived PWK/PhJ (PWK) as resilient to A β -induced cognitive deficits to determine how synaptic health in vulnerable neuronal circuits was affected across these divergent strains. Since age is the primary risk factor for AD, I determined the impact of genetic complexity on synaptic health in neuronal circuits in the prefrontal cortex that are known to be vulnerable during aging (**Chapter 3**) to discover that PWK are resistant to age-related synapse loss. Finally, since B6 and PWK mice that carry a transgene driving AD-related A β pathology have been shown to develop different types of microglia states, I evaluated the impact of A β and microglia depletion on hippocampal synaptic health in these strains (**Chapter 4**). I found that while B6 were

susceptible to microglia-dependent synaptic destabilization that appeared to be reliant on microglia-dendrite contacts, PWK were completely resilient. Together, the data reported throughout the three results chapters in this thesis provides important evidence for the influence of genetic context on aging resistance and AD resilience, anchored in the way in which synaptic dynamics are regulated. Future work will leverage the PWK mouse strain featured here to identify the genetic and molecular mediators of their ability to maintain neural circuit and synaptic integrity during aging and AD. Studies that leverage genetic complexity in mouse models of human disorders provide greater translational promise for the eventual development of personalized therapeutics, in this case to treat AD and extend cognitive health span.

Dedication

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List of Copyrighted Materials

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List of Abbreviations

3MS – Modified Mini-Mental State Examination
5XFAD – transgenic mouse model carrying 5 familial Alzheimer’s disease mutations
A β – Amyloid beta
A β 1-42 – Amyloid beta 1-42 isoform/fragment
AAV – adenoassociated virus
AD – Alzheimer’s disease
AD-BXD – BXD mice crossed to 5XFAD transgenic mouse model
AMP-AD – Accelerating Medicines Partnership- Alzheimer’s Disease
ANOVA – Analysis of variance
APOE – Apolipoprotein E
ApoE2 – Apolipoprotein receptor 2
APP – Amyloid Precursor Protein
APP/PS1 – transgenic mouse model carrying 3 familial Alzheimer’s disease mutations
AT – Array tomography
ATP – Adenosine Triphosphate
AXL – AXL receptor tyrosine kinase
B6 – C57BL/6J mouse strain
BXD – B6 x D2 recombinant inbred panel
C3 – Complement component 3
CA1-to-PFC – projection circuit from hippocampal CA1 to the prefrontal cortex
CA²⁺ – calcium
CAA – Cerebral Amyloid Angiopathy
CaM – calmodulin
CASI – Cognitive Assessment Screening Instrument
CAST – CAST/EiJ mouse strain
CC – Collaborative Cross
CFA – contextual fear acquisition
CFC – contextual fear conditioning
CFM – contextual fear memory
CNS – Central nervous system
CR3 – Complement Receptor 3
CRISPR – clustered regulatory interspaces short palindromic repeat
CSF1 – Colony Stimulating Factor 1
CSF1R – Colony Stimulating Factor 1 Receptor
CX3CL1 – C-X3-C motif chemokine ligand 1
CX3CR1 – C-X3-C motif chemokine receptor 1
D2 – DBA2/J mouse strain
DAM – disease associated microglia
DLGAP2 – DL Associated Protein 2
DL-PFC – Dorsolateral prefrontal cortex
DO – Diversity Outbred
EC – entorhinal cortex

EGFP – Expression Green Fluorescent Protein
 EM – electron microscopy
 ES – Effect size
 fAD – Familial/Early Onset Alzheimer's Disease
 FGF2 – fibroblast growth factor 2
 GAS6 – Growth Arrest Specific 6
 GO – Gene Ontology
 GRP – genetic reference panel
 GPNMB – glycoprotein NMB
 GWAS – Genome-wide association studies
 HC – hippocampus
 IFN – Interferon
 IFNAR – Type-1 interferon receptor
 IL-1 – Interleukin 1
 IL-6 – Interleukin 6
 IL-14 – Interleukin 14
 IPA – Ingenuity Pathway Analysis
 iPSCs – induced pluripotent stem cells
 IRM – interferon responding microglia
 LOAD – Late Onset Alzheimer's Disease
 LRT – likelihood-ratio test
 LTP – Long-term potentiation
 MAP – Memory and Aging Project
 MERTK – MER proto-oncogene, tyrosine kinase
 MCI – mild cognitive impairment
 MMSE – Mini-Mental State Examination
 NHP – nonhuman primate
 NMDA – N-methyl-D-aspartate
 NOD – NOD/ShiLtJ mouse strain
 NPAM – non-plaque associated microglia
 Ntg – Nontransgenic
 NZO – NZO/HILtJ mouse strain
 OPC – Oligodendrocyte precursor cell
 P2RY12 – Purinergic Receptor P2Y12
 PAM – plaque associated microglia
 PDGF-B – platelet derived growth factor subunit beta
 PFC – prefrontal cortex
 PFC-to-RE – projection circuit from prefrontal cortex to nucleus reuniens
 PSEN1 – Presenilin 1
 PSEN2 – Presenilin 2
 PWK – PWK/PhJ mouse strain
 Q1 – quartile 1, smallest measurements in a distribution
 Q4 – quartile 4, largest measurements in a distribution
 QTL – quantitative trait locus

RE – nucleus reuniens
RELN – Reelin
RGC – retinal ganglion cell
RNAseq – RNA sequencing
ROS – Religious Orders Study
ROSMAP – Religious Orders Study Memory and Aging Project
SA – Spontaneous alternation
SC – subcluster
scRNAseq – single cell RNA sequencing
SFK – Src-family tyrosine kinase
SLM – stratum lacunosum moleculare
snRNAseq – single nuclear RNA sequencing
SNP – single nucleotide polymorphism
SO – stratum oriens
SPP1 – Osteopontin
SR – stratum radiatum
ssEM – serial section electron microscopy
TG – transgenic
TNF- α – Tumor Necrosis Factor Alpha
Touch+ – Dendritic segment with a microglia process coming into contact
Touch- – Dendritic segment with no microglia processes coming into contact
TPM – Transcripts per million
TREM2 – Triggering Receptor Expressed on Myeloid Cells 2
VLDLR – very-low-density lipoprotein receptor
WGCNA – Weighted Gene Co-expression Network Analysis
WSB – WSB/EiJ mouse strain
WT – wild-type

Chapter 1: Introduction

1.1 Cognitive aging

Aging is an unavoidable time-dependent process that affects most living organisms. Biological aging is characterized by cellular and molecular hallmarks including genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication¹. In higher order mammals (rodents, primates and humans) these cellular and molecular events often manifest in the central nervous system (CNS) through the slow but progressive loss of normal memory and cognitive functions². This is of particular interest for human populations, as average lifespan has been significantly extended over the past century, calling for a need to promote/extend health span attributes including cognitive longevity³. Age is the greatest known risk factor for dementia and other neurodegenerative disorders affecting the CNS, also calling for a need to better understand how these processes interact to culminate in functional decline. Furthermore, humans cognitively age at different rates, suggesting factors such as genetic background influence the onset and severity by which these processes occur⁴.

1.1.1 Dementia

Dementia is an umbrella term used to describe symptoms of abnormal cognitive dysfunction and occurs across many neurodegenerative diseases. According to the World Health Organization more than 55 million people worldwide are suffering from dementia. Human health span continues to increase each decade, supporting the prediction that the number of patients suffering from dementia and related disorders will double by the year 2040⁵.

1.1.2 Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder caused by abnormal damage to neurons in the brain. AD affects an estimated 6.7 million Americans that are aged 65 and older, and anticipated to grow to 13.8 million by the year 2060⁵. AD was first described in 1906 by Dr. Alois Alzheimer, who was treating patient Auguste Deter, an individual exhibiting midlife cognitive dysfunction⁶. Upon autopsy of Deter, it was discovered the patient's brain contained abnormal protein aggregates which are now commonly known as amyloid beta ($A\beta$) plaques and hyperphosphorylated tau tangles. While $A\beta$ plaques are found in the extracellular zone, tau tangles manifest intracellularly and primarily in neurons. While tau is a common pathology among several neurodegenerative disorders (termed tauopathies), $A\beta$ plaques are a specific pathological hallmark of AD. Importantly, these accumulated pathologies, as well as the cellular and molecular events that accompany them, begin decades before dementia symptom onset, making the need for identification of the understanding of early events in AD much more necessary⁷.

AD progresses through three clinically-defined stages: an early, preclinical stage with no cognitive symptoms, a middle stage in which patients exhibit mild cognitive impairment (MCI), and a final stage that is marked by severe dementia symptoms often affecting learning and memory and executive functioning⁸ (**Figure 1.1**). Patients will enter the clinic with a description of abnormal memory loss (described by the patient themselves, family members or caregivers) accompanied by impairment of at least one additional cognitive domain such as attention, language, visuospatial skills or problem solving⁹. A clinician will employ multiple techniques to diagnose the disease, beginning with standardized tests of memory, problem solving, attention, counting and language. The most widely used tests include the Mini-Mental State Examination (MMSE),

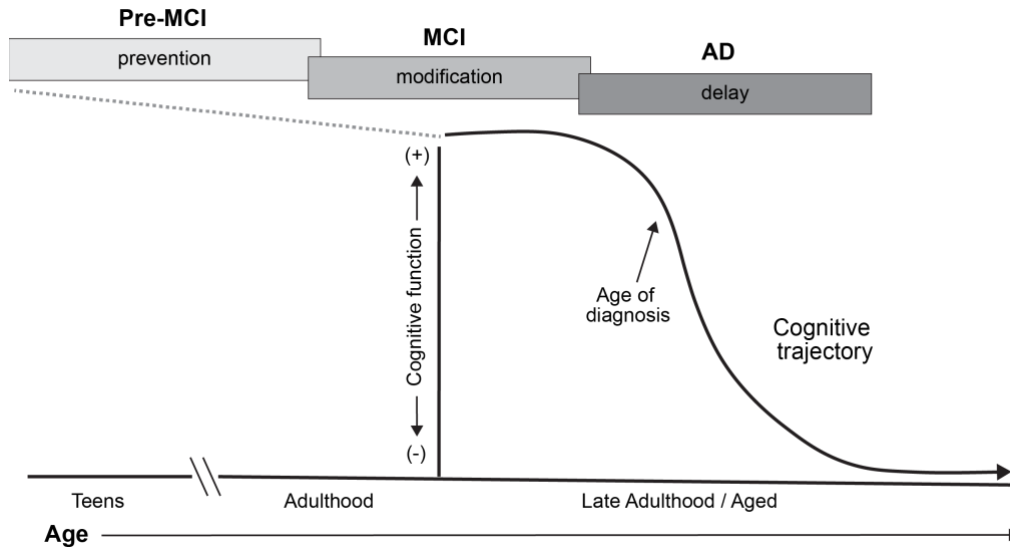


Figure 1.1: Trajectory of cognitive aging and dementia to AD diagnosis. Schematic of classical progression of cognitive aging as it progresses from early adulthood (pre-MCI), mild cognitive impairment (MCI) and Alzheimer's disease dementia (AD). When patients are diagnosed with AD due to loss of cognitive function and lower scores on classical cognitive tests it is well after many of the biological processes have accumulated to cause disease, many of which are irreconcilable. Adapted with permission from Mufson *et al*, *Frontiers Neuro*, 2019.

modified MMSE (3MS) and Cognitive Assessment Screening Instrument (CASI), which can elicit basic information about key cognitive abilities during a clinician appointment¹⁰.

The primary risk factor for developing AD is an age of 65+ years, but other risk factors have been identified such as genetic variants/mutations and biological sex, among others^{11–13}. Since 60% of AD patients are female, there has been an increased demand for understanding the sex-specific mechanisms of the disease, especially since historical studies in humans and mammalian model organisms have only utilized male subjects^{14,15}. In addition to sex, genetic factors are a primary determinant of AD risk and severity. Rare mutations in the amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) genes account for 71% of patients that develop Early Onset AD, which is also known as Familial AD (fAD) due to the autosomal dominant inheritance pattern in affected families¹⁶. However, fAD accounts for 5% of the total AD patient population, suggesting additional genetic components influence symptomology of the

more common late onset AD (LOAD)¹⁷. Human genome-wide association studies (GWAS) have identified common variants in genes such as apolipoprotein E (APOE) to increase the risk of developing LOAD^{18,19}. Intriguingly, many of the genes the variants are within or proximal to are primarily expressed in microglia, the resident immune cells of the brain, which are thought to be major contributing factors to AD susceptibility, onset and severity as activated microglia are a hallmark AD pathology^{20,21}.

In addition to known genetic risk, a recent concept surrounding AD is that certain individuals harbor factors that promote resilience to dementia symptoms. This has stemmed from an observation that patients carrying high-risk fAD mutations exhibit variation in the age at which clinical symptoms manifest, suggesting additional factors in their genetic background cause increased resilience to disease onset²². Several studies have identified mutations in the Reelin (*RELN*) gene as well as the *APOE3* Christchurch mutation to promote resilience to AD pathologies, providing evidence for these genetic factors^{23,24}. Additional findings have supported that undiscovered genetic components promote AD resilience, as subsets of cognitively intact elderly individuals have been found to carry substantial amounts of pathological A β plaque burden (and tau pathology) at autopsy²⁵. Model systems in which subjects carry known risk for developing AD pathologies are critical to uncover novel resilience mechanisms.

1.2 Modeling AD in mice

Mice are the most used animal for studying human disease, as they carry many biological similarities to humans. Mice can be genetically manipulated, have shorter lifespans which allow for studies of aging-related disorders, and are cost-effective due to their small size and large reproductive capacity²⁶. Mice have been used in biomedical research historically for almost a century, an example being The Jackson Laboratory,

which was established in 1929 as one of the largest repositories for mouse models of disease. Several types of genetic mouse models are traditionally used to study human diseases, including transgenic and knock-in lines. Transgenic mouse models require the introduction of a foreign gene (known as a transgene) into the mouse genome in the germ and somatic cells, which causes the expression and propagation of the transgene to future generations²⁷. Transgenic models are a tool used to overexpress a mutant form of a gene that is thought and/or known to be involved in a disease. While transgenic lines are useful for disease research, they are often considered artificial, and many times are inserted randomly into the mouse genome which can have unknown disruptions and consequences to the phenotypes (both disease-related and unrelated) being measured.

To combat the weaknesses of transgenic lines, knock-out/knock-in mouse lines allow for specific mutations to be made precisely within a gene of interest to either remove the ability for the gene to be expressed (knock-out) or manipulate the gene to make it identical to the human gene and/or carry known human risk variants (knock-in)²⁸. While overcoming the limitations of transgenic models, knock-in models often take longer to manifest disease pathologies. Developments in CRISPR/Cas9 technology have allowed for knock-in/knock-out models to become more widely explored across model organisms. CRISPR (clustered regulatory interspaced short palindromic repeat) is a gene editing tool that leverages custom designed guide RNA probes that are complementary to the targeted DNA sequence, and the Cas9 protein causes DNA strand breaks at the targeted location²⁹. After the breaks are made to the targeted DNA, the natural cellular machinery repairs the damage. When applied alongside a new DNA template provided to the cell, the introduction of a new gene or mutation (knock-in) into the host genome can be done. Technologies such as CRISPR-based gene editing have allowed for the development of new mouse models to study the effects of human-relevant AD mutations while avoiding limitations of traditional transgenic models^{30,31}.

1.2.1 fAD models

The most commonly used mouse models of AD are transgenic lines that overexpress humanized *APP* and/or *PSEN1*, and harbor mutations that cause fAD in humans^{32,33}. These models are often used to study the deposition of A β plaques since it is the unique hallmark neuropathology in AD. There are many differences amongst these models including the promoter driving transgenic expression, and the background strain by which they were developed and maintained on³⁴. Alternative promoters are an important consideration because they can drive transgene expression (and therefore A β pathology) in alternative cell-types and tissues that may not be desired. For example, platelet derived growth factor subunit beta (*Pdgf-b*) is a commonly used promoter to drive transgenic expression in neurons, but is also expressed in heart and lung tissue causing possible tissue-response effects outside of the CNS³⁵. This has called for a promoter expressed in mature neurons, *Thy-1* (CD90), which is more commonly used in recent transgenic models³⁶. The genetic background of AD mouse models had been underappreciated, as many have been developed on unknown or mixed strain backgrounds. Mouse genetic background has been shown to strongly influence complex phenotypes such as learning and memory, and anxiety³⁴. For example, C57BL/6J (B6) mice exhibit enhanced performance on Morris water maze compared to other commonly used laboratory strains 129/Sv (129) and DBA/2J (D2)^{37,38}. Since strain is a major driver of variation in disease phenotypes, mouse models on mixed genetic backgrounds often report conflicting results, as each cross to an inbred strain creates a variation of unknown genetic factors that can worsen or prevent decline of measured output³⁹. While this does not diminish the use of mouse models for the study of AD, it does create a

need to have a better understanding of genetic mechanisms that influence disease severity in both mice and humans.

The mouse models leveraged in this thesis were two commonly studied strains relevant to fAD: the 5XFAD⁴⁰ and *APP/PS1*⁴¹ transgenic mouse models of severe amyloidosis. 5XFAD mice express human *APP* and *PSEN1* transgenes that carry a total of five fAD-linked mutations: the Swedish (K670N/M671L), Florida (I716V) and London (V717I) in *APP*, and M146L and L286V in *PSEN1*⁴⁰. These mice rapidly accumulate high levels of A β ₁₋₄₂ beginning at 1.5 months of age, with parenchymal plaque deposition at 2 months of age, making it one of the earliest onset and severe models to study for AD. The *APP/PS1* model expresses human *APP* and *PSEN1* transgenes that carry two fAD-linked mutations: the Swedish *APP* mutation and *PSEN1* mutation lacking exon 9 (dE9)^{42,43}. *APP/PS1* mice are a much less aggressive model when compared to 5XFAD, where A β plaques emerge in the parenchyma at 4 months of age, progressively increasing until exponential plaque growth at 8 months of age⁴⁴. While initially generated on mixed/unknown strain backgrounds, both 5XFAD and *APP/PS1* models are maintained on the laboratory inbred B6 genetic background, providing researchers the proper mouse genetic tools that are not confounded by mixed strain effects. Furthermore, both models exhibit AD-relevant phenotypes at a hemizygous genotype, which was important for experiments conducted here to study the contribution of genetic background since there is no need to create homozygous progeny.

1.2.2 Mouse genetic reference panels to map complex traits

All mouse models discussed up until this point have been generated and studied on either a single laboratory inbred (typically B6) or mixed genetic background of unknown allelic combinations. Due to the high heritability of high-risk fAD mutations, as well as

genetic influences of resilience to LOAD, mouse genetic background has become more appreciated in recent decades (**Figure 1.2**). One of the first intercross studies leveraged two laboratory inbred mouse strains, the B6 and D2, carrying the R1.40 *APP* transgene to drive pathological A β accumulation. Quantitative trait locus (QTL) mapping was performed on the intercross progeny to identify three loci on chromosomes 1, 2 and 7 that controlled observed differences in A β levels between B6 and D2 mice⁴⁵. This finding suggested that genetic background controls AD-relevant phenotypes, including the pathological deposition of A β plaques, without altering the expression of the transgene that drives the pathology. Studies like these have paved the way for more complex population- and strain-based analyses to identify how genetic background influences AD-relevant characteristics.

Extending beyond traditional intercross studies, genetic reference panels (GRPs) have been developed for mapping complex phenotypic traits to identify regions of the genome regulating their activity. The GRP most common in mouse studies of this nature are the BXDs, which is a family generated over 50 years ago and derived from an initial cross between the B6 and D2 inbred mouse strains^{46,47}. To date there are 140 genetically distinct BXD strains, which accounts for the 5 million SNPs between the two founding lines that can be leveraged for studying complex phenotypes often observed in aging and neurodegeneration. The BXDs have been crossed with the transgenic 5XFAD mouse strain to study how genetic variation impacts AD-relevant phenotypes (including cognitive measures) in mice harboring large amounts of A β plaque pathology. This study found that performance on contextual fear conditioning and y-maze behavioral assays was highly heritable, corresponding to differences in LOAD polygenic risk⁴⁸.

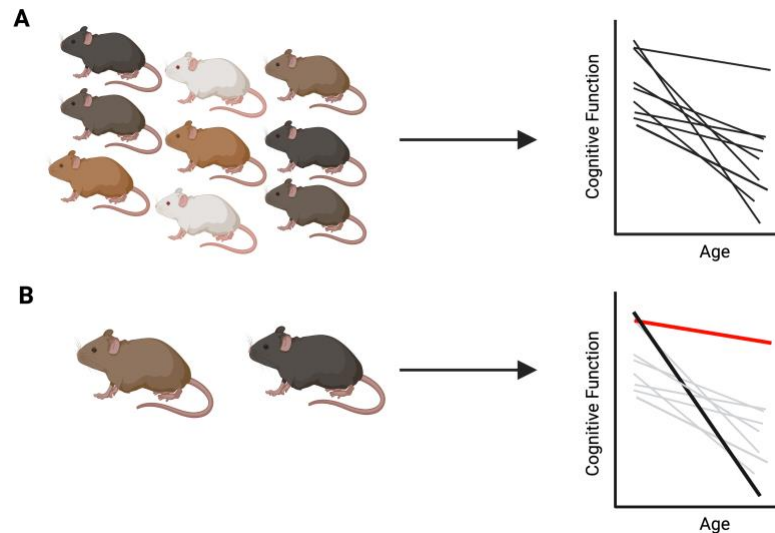


Figure 1.2: Leveraging genetic diversity in mouse models of aging and AD. Inherent genetic diversity of mice can be leveraged in AD and aging studies using two primary ideologies: (A) Represents mouse populations that have been established to study phenotype heterogeneity, such as cognitive function, complementary to how human populations are studied. (B) Identification of two distinct mouse strains that exhibit phenotypic diversity or extremes, as exemplified by red (resilient) and black (susceptible) linear regressions for measures of cognitive function. This is similar in concept to identifying resilient and susceptible human individuals to study biological factors that promote healthy aging and resistance to developing AD. Figure created with BioRender.

Recently developed mouse GRPs are the Collaborative Cross (CC) and Diversity Outbred (DO) multi-parental populations^{49,50}. Both panels are similar to the BXD but derived from eight parental strains (that carry many more SNPs across their genomes⁵¹) instead of two. The combination of multi-parental panels can be leveraged to study allelic effects on complex phenotypes with a higher mapping resolution. A strength of these lines is they harness a greater degree of genetic diversity by the incorporation of parental strains part of sub-species outside of *Mus musculus domesticus* that include *Mus musculus castaneus* (CAST/EiJ) and *Mus musculus musculus* (PWK/PhJ). Additionally, several parental lines already show susceptibility to common human diseases such as obesity (NZO/HILtJ), type 1 diabetes (NOD/ShiLtJ), and cancer (A/J), making their CC and DO progeny an excellent resource to study modifiers of human

disease. The DO population has been used to map a genetic modifier of working memory decline with age to identify the DL Associated Protein 2 (*Dlgap2*) gene as a candidate for improving cognitive function during normal aging⁵². However, since these mice are relatively new, there are currently limited studies on their use in neurodegenerative disease research combining these models with known AD pathologies, leaving future modifiers to be identified and explored.

1.2.3 Diverse mouse strains to study aging and AD complexity

The genetic diversity of individual mouse strains is a tool that has been harnessed for studying individual differences in AD phenotypes. Instead of crosses that generate large populations of diverse mice, these studies leverage the inherent genetic complexity across inbred laboratory and wild-derived mouse strains to identify genetic backgrounds that replicate AD resilience and susceptibility phenotypes (see **Figure 1.2**). The foundational study for this work crossed the B6.*APP/PS1* transgenic fAD model to wild-derived inbred strains CAST/EiJ (CAST), PWK/PhJ (PWK) and WSB/EiJ (WSB) to evaluate how genetically distinct backgrounds influenced divergence on AD phenotypes⁵³. One finding was that *APP/PS1* mice on the WSB genetic background showed greater susceptibility to AD neuropathologies, including neuronal cell loss and cerebral amyloid angiopathy (CAA), proposing WSB as a more translatable model to evaluate AD susceptibility mechanisms, particularly in neurovascular dysfunction. Importantly, and relevant to the current thesis, this study also highlighted that despite showing robust microglia activation and plaque deposition, PWK.*APP/PS1* mice failed to exhibit cognitive deficits (compared to B6 counterparts), nominating the PWK as a strain to study mechanisms of AD resilience.

A current debate surrounding AD mouse models is they do not reflect the human disease, with specific interest concerning the conservation of microglia states. To

address this single cell RNA sequencing (scRNAseq) was performed on isolated microglia from the same *APP/PS1* wild-derived mouse strains described above, at the same 8 month age analyzed previously^{53,54}. They found that mouse strains developed heterogenous microglia states, especially those commonly characterized in AD such as disease associated microglia (DAM) and interferon responding microglia (IRM)⁵⁴. This suggested that while exhibiting similar levels of overall microglia activation to A β pathology, genetic context may influence the functional impacts of microglia on the surrounding brain microenvironment. Additionally, genes differentially expressed in wild-derived CAST, WSB, and PWK compared to laboratory inbred B6 across the microglia states reflected greater concordance with scRNAseq studies from human AD patient tissue. These findings together, as well as work currently being conducted leveraging multiple models, suggest that the inclusion of diverse genetics into AD mouse models may be a key to greater translational capacity and clinical relevance. Additionally, findings from diverse AD mouse models will pave the way for more conclusive testing of microglia-specific targets to improve cognitive and neuropathological outcomes for AD patients. Finally, the inclusion of diverse model systems, such as population-based approaches versus the identification of resilient and susceptible individuals (as depicted in **Figure 1.2**) provides a robust understanding of the neuropathological mechanisms that drive disease severity, as well as the ability to perform more high-resolution functional testing, which is demonstrated in this current work.

1.2.4 Behavioral assays to assess cognitive performance in mice

The primary objective for evaluating how an individual is displaying dementia symptoms is through standardized approaches to measure cognitive performance¹⁰. To align mouse models to human studies, assays have been developed that harness

several instinctual aspects of mouse behavior and test their performance when they are impaired through insults such as aging and/or AD-linked genetic and pathological risk. Several factors are important for determining which assays properly test for cognitive deficits, such as cognitive domain (working, short-term or long-term memory), confounding factors such as motor or sensory deficits that could be induced in the tested models, as well as if the assay is to be performed longitudinally or cross-sectionally over multiple cohorts⁵⁵. One of the more popular approaches to test multiple behavioral measures in animal models (although not leveraged here) is the Open Field assay. This task assesses rodent locomotion, exploratory behavior, and multiple anxiety-related domains⁵⁵, which are all known to be affected during aging and AD. During this test, the rodent is placed in a square-shaped open field arena and monitored by camera and infrared photobeam sensors to calculate the number of times the mouse crosses the open field both vertically and horizontally (**Figure 1.3**). This assay captures a rodent's innate desire to hide when feeling threatened or anxious, therefore an important aspect is determining the amount of time spent at the exterior (more anxious) compared to central (more adventurous) points of the maze⁵⁶. Furthermore, motor function deficits or hyperexcitability behavioral phenotypes can be detected through measures of general locomotor function gathered throughout the testing period⁵⁷.

A relatively high-throughput assay that captures general locomotion and cognitive performance (typically the working memory domain) is Y-maze spontaneous alternations test⁵⁵. To perform this, a Y-shaped maze, which is a clear polycarbonate arena constructed in the shape of a Y, is placed on a table with 3 different visual cues taped on the walls at the end of each arm of the maze. The mouse is placed in the maze and allowed to explore for approximately 8-10 minutes, which harnesses the innate tendency for mice to explore new spaces (**Figure 1.3**). Therefore, when a mouse completes a correct spontaneous alternation, it will explore an arm of the maze, and then move to the

arm of a maze that it hasn't explored in the previous alternation⁵⁸. Since this test measures spatial and working memory and is relatively high-throughput in nature, it can be utilized longitudinally to assess general memory decline over time⁵⁹.

The Y-maze task has been adapted many ways in order to add delay periods and different types of alternations, which include the T-maze⁶⁰ and the delayed-spatial (or novel spatial recognition) task. This task is an adapted approach from the Y-maze and novel object recognition task⁶¹ which assesses both working and short-term memory. For this test, the Y-maze is adapted to have 3 visual cues moved from the walls surrounding the apparatus to be directly at the end of each arm of the maze (**Figure 1.3**). This is a two-part test, in which the first part the rodent is placed in the maze and allowed to explore two arms (start and familiar) with the third arm (novel) blocked with an obstacle. Results from the first trial are used to ensure the rodent spends relatively equal amounts of time (>30%) in each arm and removed from further analysis if it does not. After the first trial the rodent is placed in its home cage for a delay period (typically ranging from 10-60 minutes), after which the obstacle from the novel arm is removed and the mouse is placed back into the maze and allowed to explore all three arms^{53,55}. Results from the second trial are used to calculate the percentage of time spent in each of the three arms of the maze, and a mouse with adequately functioning short-term memory will have spent more time in the novel arm compared to the start and familiar arms. This test is sensitive to both age-related or pharmacologically-induced cognitive deficits in young male B6 mice⁵⁵, and has also been found to measure behavioral differences across genetically distinct AD mouse strains⁵³.

Additional tests exist that are more complex and measure different aspects of mouse behavior that will not be covered in this introduction, including Morris water maze and Barnes maze^{62,63}, among others. However, a final, task that was heavily utilized in **Chapter 2** of this thesis is the contextual fear conditioning (CFC) paradigm. There are

many iterations of this test, but the version leveraged here was an adaptation to be performed in a more high-throughput nature⁶⁴. CFC is an associative learning test that is presented in the form of a cue (visual, sensory, auditory) and/or context (in this case a physical context) and measures a form of fear-based Pavlovian response⁶⁵. In the iteration of this task used here, rodents were tested over two days with two trials. During the first trial the rodent is placed in a CFC chamber (which has a distinct context) and administered a foot shock four times. Since a mouse's response to being shocked or scared is to freeze⁶⁶, the amount of time the mouse spends freezing after each shock is measured, which typically appears as some form of positive linear regression, and reported as fear acquisition (CFA). The second trial, which is administered 24 hours after CFA, is performed by placing the mouse back into the chamber and freezing measured without administering any foot shocks. The amount of time the mouse spends freezing during the second trial, termed fear memory (CFM), is considered a measure of long-term memory performance since it required the rodent to recall and associate the current environment with the shocks it received the day prior (**Figure 1.3**). Rodents that exhibit more freezing behavior on the CFM trial are considered to have better long-term memory performance than mice that freeze less⁶⁴. Since the CFM and CFA assays have been shown to directly involve the hippocampal and prefrontal cortical brain regions, they are considered valuable for assessment of age- and AD-related cognitive deficits in rodents that can translate back to human studies⁶⁷. Behavioral studies are only a critical component to **Chapter 2** of this thesis, but it is important to understand how they can be leveraged for translational relevance, their deficits, and the types of cognitive domains they capture, especially in dementia-related diseases. Furthermore, there are limitations to mouse behavioral testing, including the large amount of mouse-to-mouse variability in performance across all tasks mentioned here, and the impact of environmental components to behavioral tasks. Critically to this thesis, traditional behavioral assays

have only been developed for laboratory inbred strains, requiring additional testing and validation for the employment of these tests on wild-derived mice to ensure they are testing the cognitive domain and neural circuit in question.

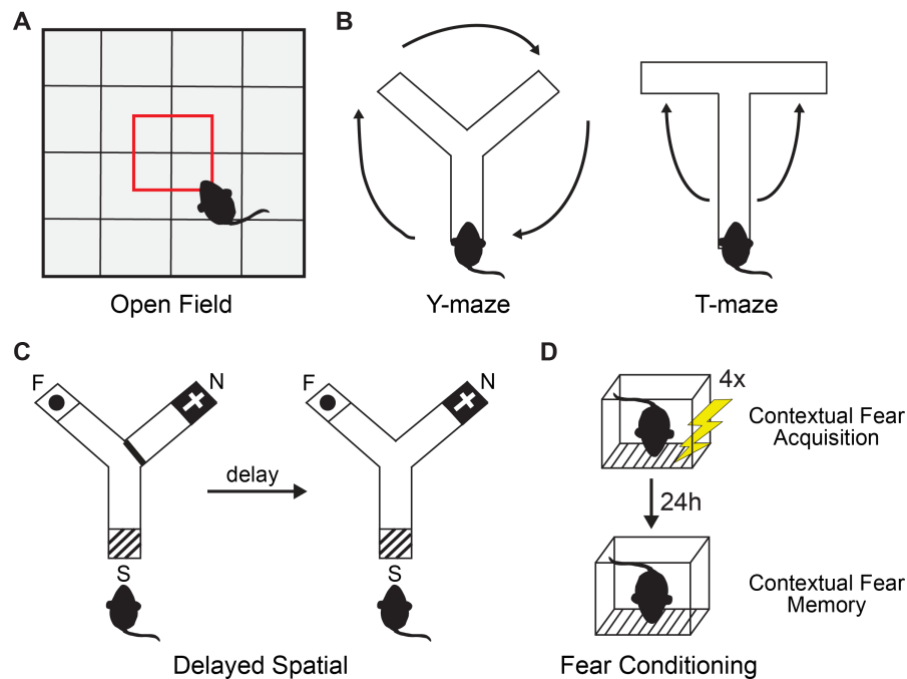


Figure 1.3: Behavior assays to measure different components of mouse cognition. Overview of behavioral assays used to measure mouse cognition (most relevant to the results in this thesis). (A) Open field assay design to measure multimodal elements of mouse behavior including anxiety and exploratory measures. (B) Assays to measure working memory performance through spontaneous alternations on Y-maze (left) and T-maze (right). (C) Delayed spatial assays leveraged across mouse strains for working memory and short-term memory performance. S = start, F = familiar, N = novel. (D) Adapted contextual fear conditioning assay which measures short-term and long-term memory in mice. Short-term memory is assessed during fear acquisition tests, and long-term memory measured during contextual fear memory test performed 24 hours after acquisition test.

1.3 Cellular and molecular components of aging and AD

Recent developments in sequencing technologies have led to discoveries on the molecular processes (indicated by changes in RNA transcript abundance) occurring in AD patient brains at the time of death⁶⁸. These include bulk RNA sequencing (RNAseq) performed on a tissue sample or cell population, and single cell or single nuclear

RNAseq (scRNAseq/snRNAseq) which individually sequences isolated cells or nuclei⁶⁹. Application of these technologies in translatable mouse models of aging and AD have allowed for the identification of early molecular signatures that are predictive of the latter stages of disease progression⁵⁹. Combined with sophisticated computational approaches that include differential expression between groups and gene co-expression network analysis^{70,71}, there have been breakthroughs in the identification of the cell-types and pathways that can be targeted to delay aging- and AD-related cognitive decline. This thesis leverages a variety of these tools and techniques to uncover how genetic context dictates the way in which cells interact in the CNS to drive aging and AD. This section will serve as an overview of several of these key studies and how findings have influenced the study of mechanisms underlying cognitive aging and AD.

1.3.1 Studies in humans identify aging- and AD-related molecular signatures

As aging is the primary risk factor for developing neurodegenerative disorders such as AD, several studies have been conducted to understand the pathways that become disrupted during normal aging that are exacerbated in AD. Common mechanisms that occur in brain aging are centered around neuronal circuit disruption and include synaptic atrophy, dendritic branch retraction on pyramidal neurons, abnormalities in the cellular cytoskeleton, as well as hallmark increases in neuroinflammation that include reactive astrocytes and activated microglia¹. Since brain aging hallmarks are centered around the disruption of neuronal circuits critical for memory function⁷², many studies have focused on neural mechanisms that drive this. Additionally, chronic inflammation is another hallmark thought to progress with aging, and when targeted has been shown to improve cognitive deficits in mice⁷³. However, most of the human-based research in aged populations has been to identify the mechanisms that differentiate normal aged individuals from those that develop neurodegenerative disorders such as AD.

Insights into the causal genes and pathways that increase risk for AD have been elucidated through human GWAS^{18,20,74}. Recent studies have moved a step forward to employ sequencing techniques on postmortem brain tissue samples and evaluate how factors in one's genetic background promote dysregulation of molecular networks and cell-types to cause AD compared to individuals without dementia. To evaluate these processes, several large-scale longitudinal human studies have been established that enroll volunteers who are monitored from late adulthood (to establish a baseline) until death (the degree of which varies based on study). Participants part of these studies have agreed to donate their postmortem brains among other terminal tissues for continued study. U.S.-based studies include the Religious Orders Study (ROS), Rush Memory and Aging Project (MAP)^{75,76} and the Accelerating Medicines Partnership-Alzheimer's Disease (AMP-AD)⁷⁷.

Findings from these cohorts have paved the way to a better understanding of the processes driving AD, especially with the molecular evaluation of postmortem brain tissue. Using bulk RNAseq of isolated dorsolateral prefrontal cortex (DL-PFC) from individuals in the ROSMAP study, several gene co-expression networks that were predictive of AD cognitive decline were identified⁷⁸. The module identified as the most strongly correlated to cognitive decline, m109, was weakly correlated with postmortem amyloid and tau load, separating the impact of cognitive symptom severity and AD pathology. Genes in this module were primarily expressed in brain microglia, which are thought to be the drivers of neuroinflammation observed as a pathological hallmark of AD. In parallel to this study, bulk RNAseq analysis across multiple brain regions (including DL-PFC) from the AMP-AD consortium identified several transcriptional networks commonly enriched in human AD patients (and across brain regions)⁷⁷. These modules, which were conserved in traditional AD mouse models including the 5XFAD, were enriched for functions associated with synaptic and neuronal dynamics, as well as

microglia and inflammation, further nominating the two key processes that warrant further study in AD.

Beyond bulk RNAseq which is valuable for identifying pathways and nominating general cell types associated with disease stages, recent developments in single cell sequencing technologies (scRNAseq and snRNAseq) can quantitatively analyze how cell compositions are different in AD patients compared to non-AD patients. These studies have further nominated increased microglia as a primary hallmark of the human AD brain, and in particular microglia that associated with A β plaques (DAM)⁷⁹. Additionally, these studies sub-divide neuronal populations (excitatory, inhibitory, sub-region) beyond what can be accomplished through computational deconvolution of bulk RNAseq, which allows for the identification of smaller neuronal populations that are disrupted in AD as well as molecular pathways that are altered within these cell-types. However, a major caveat is the limitation of the acquisition of postmortem tissue (often from AD patients that have exhibited symptoms for years) which does not make it possible to identify processes that are driving pre-symptomatic disease activity. This further emphasizes the importance of translatable preclinical AD models, as well as the integration of cross-species datasets that are being generated from both mouse and human tissue.

1.3.2 Complementing the brain transcriptome of AD patients with mouse models

As discussed in previous sections, a challenge to many mouse models of AD is the lack of translatability to the human condition. This raises the primary purposes of studying brain transcriptomes from mouse models: 1) evaluate how current models replicate what is observed in human studies; 2) identify what is lacking in current models that can be improved in more refined mouse models of AD. This has been a focus of the

AMP-AD human study, which identified several transcriptomic gene expression modules that are enriched in human patients, and can be used to evaluate brain transcriptomes of AD mouse models^{77,80}. Through these studies the brain gene signatures that exhibit the greatest overlap across mouse models and human AD are associated with microglia-associated inflammation and neuron-associated synaptic pathways.

An additional use of brain transcriptome studies from AD mouse models has been to identify the processes that predict late-stage disease phenotypes such as cognitive decline and A β deposition^{53,59}. It is important to understand these early processes as the primary limitation of using postmortem human brain tissue is that it is acquired often well after symptom onset, and the cascades of events that occurs by the time patients reach the clinic are irreconcilable⁸¹. While limited, these studies have found that early processes that occur in these models are associated with immune and neuronal function, mRNA and protein binding, as well as myelination⁵⁹. These findings suggest that brain immune cells (microglia) and neurons are dysregulated at early stages and warrant further investigation into the mechanisms by which they interact during early disease stages that predicts late-stage progression. This is the primary synthesizing premise of all results chapters in this thesis, to identify pathways and cell-types that predict cognitive outcomes across a diverse population, then use precision AD mouse models to test them, with a specific focus on microglia and excitatory neurons.

1.4 Microglia

1.4.1 Microglia and their role in the CNS

Microglia are the primary resident immune cells of the CNS that make up around 5-10% of the total tissue composition, depending on brain region and functional/disease state⁸². Microglia originate from a different population compared to all other neural

progenitors, arising from the yolk sac during early developmental timepoints and migrating to the CNS later (mouse embryonic day 9.5)⁸³. Since the brain is considered to be an immune-privileged organ, meaning access of the immune system is limited and under high control in order to protect neural cells, microglia are critical for their protective function which also makes them sensitive to extracellular stimuli⁸⁴. Under homeostatic conditions, microglia are actively surveying the brain microenvironment for anything that may resemble an infection, dead/dying cell, or extracellular debris so they can rapidly evoke an inflammatory response by releasing extracellular cytokines such as Interleukin 6 (IL-6) or Tumor Necrosis Factor Alpha (TNF- α), or engulf the debris and process it through an internal lysosome/proteosome system⁸⁵. Outside of their immune surveillance roles, microglia are critical for maintaining the brain microenvironment through their interactions with neuronal synapses⁸⁶. In this section, the role of microglia will be discussed as it relates to neuronal/synaptic homeostasis, and more critically how these interactions become dysregulated in aging and disease, a process studied throughout this thesis.

1.4.2 Microglia shape synaptic connections during development

A critical role for microglia during development (and even into adulthood) is pruning and shaping neuronal circuits by engulfing unnecessary or malfunctioning synapses⁸⁷. This was initially discovered through studies in the mouse visual system, particularly in the way in which microglia exhibit activity-dependent synaptic pruning via originating signals from developing neurons^{88,89}. In the mouse visual system (and now understood in the CNS) microglia-neuron crosstalk is highly dependent on ligand-receptor binding interactions including complement (C3-CR3) and CD47-SIRP α , among others^{88,90}. Microglia are known to be regulated by retinal ganglion cell (RGC) activity to prune

synapses in the developing visual system, which eventually allows for proper specification of RGC projections to ipsilateral and contralateral positions^{88,91}. C3-CR3 interactions are crucial for this specification, as mice that are deficient in either of these molecules exhibit increased RGC synaptic densities and a failure to specify to proper projection pathways⁸⁸. The CD47-SIRP α interaction axis plays an alternative role to C3-CR3 in microglia-neuron crosstalk, as mice deficient in either of these molecules exhibit increased microglia-mediated synaptic pruning in RGC inputs during postnatal development that is preferentially localized to more active RGC inputs, suggesting neuronal activity regulates this pathway⁹⁰.

Outside of the visual system, these studies have replicated into the developing brain to identify more critical microglia-synaptic interactions including the fractalkine signaling proteins, C-X3-C motif chemokine receptor 1 (CX3CR1) and C-X3-C motif chemokine ligand 1 (CX3CL1). Mice deficient in the microglia receptor CX3CR1 show increased dendritic spines (a structural indicator of a presynaptic connection, described more thoroughly in later sections⁹²) in the CA1 region of the hippocampus during P14-P21 developmental stages which corresponded functionally to a greater number of immature synapses and a reduction of overall microglia⁹³. Additionally, the microglia innate immune receptor TREM2 is critical to neurodevelopmental synaptic refinement in the hippocampus, as *Trem2*-deficient mice have reduced microglia activation and synaptic refinement in the postnatal hippocampus that translates to impairments in social behavioral tasks such as marble burying and grooming into adulthood⁹⁴. Together these seminal papers in the developing mouse have proposed a significant contribution of microglia in creating and maintaining neuronal circuits in both the visual system and brain (**Figure 1.5**).

1.4.3 Microglia maintain synaptic connectivity under homeostasis

Until recently microglia were not thought to play as significant of a role with maintaining neuronal circuitry and synaptic connectivity in the adult brain. Electron microscopy experiments have indicated microglia engulf synapses by finding that pre- and post-synaptic material was inside lysosomes of microglia from adult mouse brain⁸⁹. It is now commonly accepted that microglia are crucial regulators of neuronal homeostasis and plasticity through their phagocytic roles by collecting apoptotic cellular debris, as well as pruning weak or dysfunctional synaptic connections. More complex *in vivo* two-photon imaging studies have found that when microglia are homeostatic in the adult brain, they constantly survey the microenvironment through their processes, ready to respond to insult or injury⁹⁵. Through the activity of TAM receptors and downstream kinases MER Proto-Oncogene, Tyrosine Kinase (MERTK) and AXL Receptor Tyrosine Kinase (AXL), microglia recognize and engulf apoptotic cells by recognizing secreted TAM ligands such as Growth Arrest Specific 6 (GAS6) and Protein S, as discovered through *in vivo* studies of mice deficient in TAM receptor kinases exhibit microglia that are defective in phagocytic and migratory function⁹⁶. In addition to injury responses, one of the main signals that neurons secrete to recruit microglia is Adenosine Triphosphate (ATP)⁹⁷. Live imaging of zebrafish larvae that express microglia-specific expression Green Fluorescent Protein (eGFP) found that homeostatic microglia actively contact neuronal soma via their processes, and glutamate uncaging experiments to activate neurons observed microglia recruitment to the site of activity⁹⁸. The responsiveness of microglia to neurotransmitters has also been validated beyond zebrafish models into rodent systems⁹⁹. The mechanism by which microglia respond to glutamate release occurs through the activation of N-methyl-D-aspartate (NMDA) receptors on neurons which causes the release of ATP, and engagement of Purinergic Receptor P2Y12

(P2RY12) on microglia which then causes process mobilization and extension, and subsequent feedback of activity back onto the activated neuron¹⁰⁰.

Microglia shape and prune neuronal circuitry in adult organisms through mechanisms and ligand-receptor interactions that are used in neural development (**Figure 1.5**). This has been demonstrated through studies of adult mice homozygous and heterozygous for CX3CR1 deletions have impaired hippocampal long-term potentiation (LTP), exhibiting an impairment of synaptic plasticity. Additionally CX3CR1 deficiency negatively impacts mouse contextual fear conditioning and Morris water maze tasks, suggesting that the role of microglia in maintaining neuronal circuitry is important to higher order organismal function¹⁰¹. However, the complete loss of microglia shows more detrimental than beneficial effects in adults, as mice lacking critical microglial complement proteins C1q or C3 present with excess synapses as well as spontaneous seizures^{91,102}. Together, preliminary studies of both developing and adult animals demonstrate a critical role for microglia in establishing and maintaining neuronal connectivity, which are likely to be critically impacted under conditions of cellular exhaustion observed in aging or neuropathological challenge.

1.4.4 Microglia in normal aging

Unlike cells of the systemic immune system that do not live past a few days to several weeks, microglia slowly renew themselves at a rate of 28% annually in the human brain¹⁰³. This can be affected by conditions that promote cellular stress such as aging and neurodegeneration. During the process of aging, microglia lose their normal homeostatic functions through processes of cellular exhaustion and senescence¹. In the aged brain, microglia adapt abnormal morphologies that include the retraction of their usual ramified processes, intercellular beading, and the formation of spheroids and fragmentation of the cytoplasm¹⁰⁴. These morphological phenotypes are thought to

correspond to an abnormal function, often termed dystrophic, which is suggestive of a loss of homeostatic surveillance and possibly a primed or even sustained inflammatory response¹⁰⁵ (**Figure 1.5**). This results in a dampened migratory response and heightened proinflammatory attack when encountering an unrecognized stimulus, explaining why there are higher measures of inflammation present in the aged versus young brain. In fact, in aged individuals microglia exhibit reduced phagocytic activity and increased reactive oxygen species production, resulting in the inhibited ability of microglia to perform normal synaptic pruning and shaping mechanisms that are necessary in adult organisms¹⁰⁶. However, other studies in aged mice suggest that microglia adapt their intracellular pathways during aging to becoming more neuroprotective, which is why they appear more sensitive to extracellular stimuli¹⁰⁷. This could be very well brain and even region specific in nature, as specific neural circuits are known to degenerate more rapidly in aging than others (discussed in later sections). However, what is clear is there are differences in aged microglia from those under additional pathological stress such as in AD, which have been dissected through transcriptome analyses.

Aged microglia adapt their cellular pathways, exhibited through changes in their transcriptomic signatures. It is important to note the key molecules that have been identified to be associated with aged microglia that differentiate them from younger adults as well as under neurodegenerative conditions. This aged microglia sensome has been described in mice through bulk RNAseq studies, which is a key set of genes expressed almost exclusively in microglia when compared to other brain cells. These genes are important for neuroprotection and phagocytosis functions and include *P2ry12*, *Cx3cr1*, *Csf1r*, *Trem2*, *Cd33*, *Cd68*, among others¹⁰⁷. The traditional sensome has been found to change in the aged mouse brain by the downregulation of genes associated with functions that involve sensing endogenous ligands (*Trem2*, *Escr1*, *Tmem173*) that

corresponds with an upregulation of genes associated with functions involving host defense response, which are often extracellular or pathological challenges (*Cd52*, *Ifitm3*, *Clec7a*)¹⁰⁷. This also corresponded to a loss of purinergic receptor expression (*P2ry12*) which is classically indicative of healthy homeostatic microglia that are sensitive to neurotransmitters¹⁰⁸. Studies in aged mice have been complemented by human cortical bulk RNAseq studies that compare gene signatures in aged to middle-aged individuals¹⁰⁹. Similar to what has been found in mouse studies, the human microglia aging signature corresponds to a loss of certain cellular functions (TGF β signaling) and a gain of others (interferon signaling, antigen processing/presentation)¹⁰⁹. Together, studies in aged model organisms and humans suggest that microglia adapt a neomorphic phenotype during the process of aging, turning off certain homeostatic processes while ramping up others (**Figure 1.5**). This could be an explanation for why these cells become prone to an inflammatory response in aged-related neurodegenerative disorders such as AD, as the aged brain microenvironment has primed them to adapt a certain inflammatory cellular phenotype. However, additional factors including those inherent to an individual's genetic background may be protective of microglia transforming from a normal aging dystrophic state to a harmful inflammatory response, creating a need for future studies that differentiate aged from neurodegenerative/pathologically responding microglia.

1.4.5 Microglia in AD

In recent years there has been an exponential increase in the findings implicating microglia in neurodegenerative disorders, especially AD, where synaptic dysfunction occurs aggressively which causes eventual cognitive decline. In AD the specific challenges microglia are responding to are traditional neuropathologies, and the

accompanying secreted signals of neuronal stress and synaptic loss (as well as signals from other cells in the CNS). Microglia respond to A β plaques in AD by altering their morphology and becoming less ramified, surrounding plaques, and promoting neurotoxicity by releasing pro-inflammatory cytokines^{110,111} (**Figure 1.5**). However, this process of clearing abnormal pathology comes at the cost of neuronal synapses, as excessive synaptic loss is an early hallmark of AD that correlates with cognitive symptoms^{112–114}. Synaptic loss is thought to be mediated by microglia due to their known role in shaping and pruning neuronal circuits throughout the lifespan of an organism, as well as the microglial upregulation of synaptic pruning proteins such as those involved in the complement pathway that has been observed in AD mouse models and human tissue¹¹⁵. Additionally, depletion of microglia using the Colony Stimulating Factor 1 Receptor (CSF1R) inhibitor PLX3397 in the 5XFAD transgenic AD mouse model prevented dendritic spine loss in hippocampal CA1 neurons, as well as preventing neuronal loss in the subiculum, while leaving A β plaque pathology unaltered¹¹⁶. These initial studies implicate microglia in having a more direct role for synaptic dysfunction in AD that lies beyond A β plaque clearance.

Complement has been a more highly studied pathway in AD, with the earlier neurodevelopmental studies (discussed above) also discovering that AD mouse models have synapses that become tagged with complement protein C1q, which allows for them to be engulfed by microglia via CR3⁸⁸. A more recent study suggests that the protein Osteopontin (SPP1), which induces C1qa expression, is secreted by perivascular macrophages in the CNS and causes microglia to phagocytose A β plaques and synapses in the hippocampus of AD mouse models¹¹⁷. In fact, loss of *Spp1* in the *App*^{NL-F} transgenic AD mouse model prevented the hallmark synaptic loss that is typically observed, suggesting a possible target to prevent AD synaptic phenotypes in the

complement pathway. Other neurodevelopmental molecules have been found to be involved in microglia-mediated synaptic engulfment in AD, such as the CD47 receptor SIRP α that negatively regulates microglia-mediated synaptic pruning during development. Conditional knock-out of SIRP α in CX3CR1+ microglia in *APP/PS1* mice resulted in increased microglia-induced synaptic loss and hindered performance on open field and Morris water maze cognitive assays at 8 months of age, without altering A β plaque deposition¹¹⁸. In addition to demonstrating the role of this pathway in AD, this study also suggests that microglia-mediated synaptic pruning in AD occurs independently of the amount of A β plaque deposition.

Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) is one of the most highly expressed proteins on microglia, and a key player for microglia reactivity to A β plaques and apoptotic neurons. Early studies in primary microglia cultures showed that knock-down of TREM2 impaired the engulfment of apoptotic neurons *in vitro*¹¹⁹. More recent *in vitro* studies using differentiated microglia from human induced pluripotent stem cells (iPSCs) confirm these findings in an AD pathology-specific context, showing that TREM2 deletion inhibits the ability of microglia-mediated phagocytosis in response to A β plaques, and impairs microglia chemotaxis¹²⁰. Loss of *Trem2* in mice hinders microglia proliferation and activation in response to external challenge *in vivo*¹²¹. TREM2 is also thought to be a critical mediator in microglial activation during AD progression, particularly in the DAM state. Transgenic AD mice deficient in *Trem2* exhibit activation of a primary stage of DAM, without activating a second DAM stage that may be more responsive to plaque pathology¹²². TREM2 has also been directly implicated in synaptic pruning during aging, with complete deficiency of *Trem2* in mice showing enhanced LTP and increased dendritic spine density in hippocampal CA1 and the cortex in aged (19 months) mice¹²³.

There continue to be emerging players in the genes, proteins and pathways that regulate microglia-synaptic interactions in neurodegeneration and AD. Many of these candidates are often implicated in phagocytic mechanisms in the periphery, which can be adapted to CNS microglia when they are discovered to show high expression in CNS cell-types. CD22, a commonly known B cell receptor, was recently discovered through CRISPR/Cas9 screens to be highly upregulated on microglia from aged mice, and has critical functions in negatively regulating phagocytic activity suggesting a possible role in AD for either A β plaque clearance and/or removal of dysfunctional synaptic debris¹²⁴. The glycoprotein NMB (GPNMB) is also more recently characterized in microglia in AD, being up-regulated in a distinct microglia population in transgenic AD mice, as well as having a known role in phagocytosis¹²⁵. Here, several examples have been highlighted on critical microglia drivers of disease, but there is still much more to learn and discover about the microglia-based mechanisms that cause abnormal neuronal and synaptic function, especially during early/pre-symptomatic AD stages.

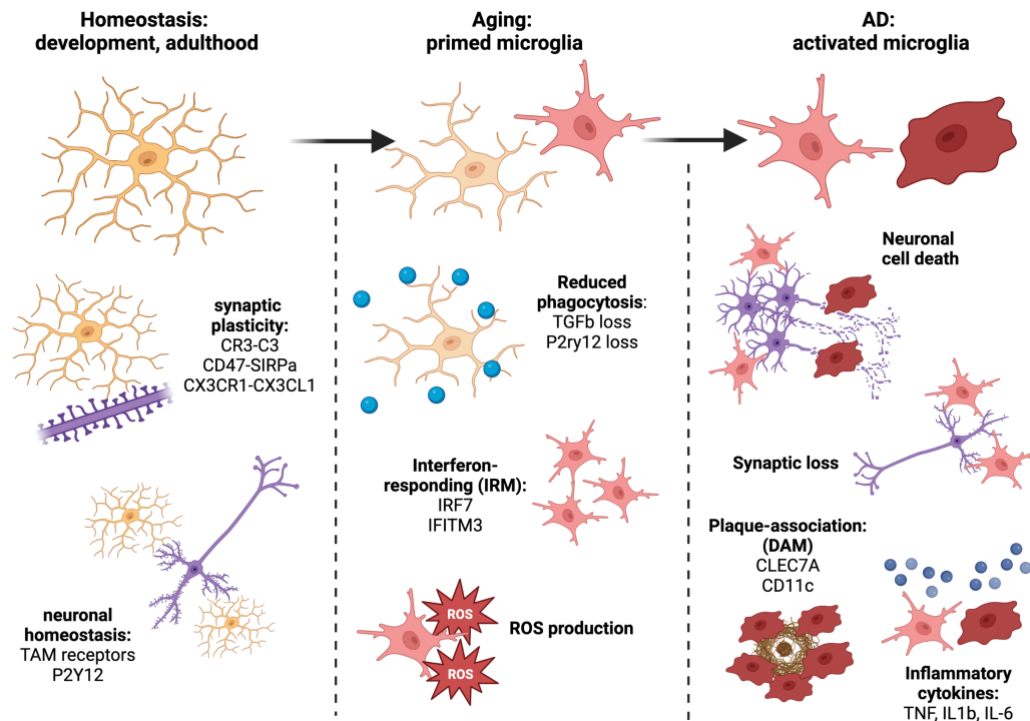


Figure 1.4: Overview transformations of microglia function and interactions with neurons throughout development, adulthood, aging and AD. Image created in BioRender.

1.4.6 Microglia states

Microglia behave similarly to their systemic counterparts (tissue-resident macrophages and myeloid cells), becoming highly plastic to adapt and respond to pathogenic challenge without proliferating. Based on the similarities between cell-types^{126,127}, it was initially thought that microglia form similar M1 pro-inflammatory and M2 anti-inflammatory functional states similar to macrophages¹²⁸. However, this theory has been contradicted through several studies, the first being the identification of the unique microglia sensome (*Cx3cr1*, *P2ry12* and *P2ry13*)¹⁰⁷. More recently, scRNAseq technologies have identified that microglia activation occurs through a continuum of states rather than semi-static subtypes or discrete M1/M2 phenotypes^{129,130}.

One of the first studies to investigate microglia at the single cell level evaluated brain microglia at different development and aging stages in a mouse lifetime¹²⁹. Here, specific states of microglia such as axon-tract *Spp1*⁺ microglia important for directing myelination during early-stage neurodevelopment, and proliferating microglia expressing cell cycle genes that were important for rapid expansion during development, were also identified in disease models used in this study¹²⁹. Importantly, this study identified and spatially characterized a population of inflammatory (*Ccl3*⁺, *Ccl4*⁺) and interferon-responding (*Ifitm3*⁺, *Irf7*⁺) microglia (IRM) that specifically arose in aged mice, corresponding to previous bulk RNAseq studies identifying these signatures in aged individuals^{107,109,129}. An important note is the state of IRM appeared to be an age-specific feature, and not necessarily as robust in the demyelinating disease models that were used in this study.

Beyond normal aging processes, microglia become activated when exposed to disease-related pathologies¹³¹. The first AD-relevant study identified the most commonly-characterized microglia state, DAM, in transgenic AD mouse models at different points of disease stages which tracked this state from early to later disease stages¹²². This DAM population was characterized through its discrete up-regulation of specific genes (*Trem2*, *Tyrobp*, *Ctsb*) and down-regulation of traditional homeostatic microglia genes (*Tmem119*, *P2ry12*, *P2ry13*, *Cx3cr1*). In addition to AD, the DAM state has been identified in other disease models including ALS, making it a signature of microglia across multiple neurodegenerative disorders rather than specific to AD¹³². In other mouse models of neurodegeneration, two additional microglia states have emerged to be secondary to the predominant DAM. These states are the aging-related IRM and MHC-II microglia¹³³.

There has been controversy over if microglia states traditionally identified in mouse models replicate in human studies¹³¹. One study found that in lieu of a DAM signature, only a few genes associated with DAM (*APOE*, *SPP1*) were up-regulated in human

microglia (using snRNAseq data)¹³⁴. There are many biological and technical factors that could hinder replication of the DAM state between the two species. One important consideration, which has been touched on in previous sections, is that human tissue represents the terminal end of a disease, while mouse studies can reflect any timepoint¹³⁵. Mouse and human studies often have diverging definitions of “control” tissues, as human studies leverage age-matched non-AD tissue, while mouse studies leverage genetically identical littermate counterparts that do not harbor high-risk AD mutations often in the form of an amyloid-driving transgene. Additionally, while classical AD mouse models arise from a single genetic background, humans are much more genetically complex, which could be why microglia states are much more diverse in human studies than initially identified in mice. In fact, individuals may harbor mutations that drive disease protective states, a concept tested in **Chapter 4. APP/PS1** AD mice crossed to wild-derived and inbred mouse strains found that microglia were much more heterogenous, with some strains such as the wild-derived PWK exhibiting a much more robust IRM response and muted DAM signature, whereas others such as the WSB fail to produce traditionally characterized activated microglia states⁵⁴. The key to unlocking the lack of mouse-to-human conservation in these states may very well be to study them across multiple mouse models with distinct genetic backgrounds. Furthermore, while these states have been identified using single cell technologies, the tools to investigate their functions have yet to be fully developed.

1.4.7 Manipulating microglia *in vivo*

To understand the impact of microglia on the adult and diseased brain microenvironment, pharmacological methodologies have been developed to deplete microglia without critically affecting the organism. A class of small molecule inhibitors for CSF1R, a receptor exclusively expressed on brain microglia and tissue-resident

macrophages¹³⁶, has shown to deplete microglia safely and efficiently (can effectively cross the blood-brain barrier) in the CNS of adult mice through an orally available formulation in chow feed^{116,137–139}. CSF1R is critical to macrophage and microglia function, and while it has two ligands that bind to it to initiate different downstream signaling responses (IL-34 and CSF1), the complete loss of CSF1R function causes the affected cells to die¹⁴⁰. One of the more popular iterations of CSF1R small molecule inhibitors is PLX3397, which was developed as a dual c-Kit (a receptor tyrosine kinase expressed on hematopoietic stem cells) and CSF1R inhibitor, with an IC₅₀ of 10 nM to c-Kit and 20 nM to CSF1R¹⁴¹. Over a relatively short period of time (7 days) and using a low dose (290mg/kg), initial studies using PLX3397 found that it will deplete brain microglia with greater than 90% efficiency¹³⁷. PLX3397 has been widely used to unravel the role that microglia play with interacting with other neuronal cells in the adult and diseased brain. One such study leveraged PLX3397 to deplete microglia after inducing hippocampal lesions (using neuronal expression of diphtheria toxin A-chain that causes robust inflammation) and found that loss of microglia during this otherwise proinflammatory post-lesion period improved recovery on cognitive tasks, and a reduction in proinflammatory cytokines in the lesioned brains¹³⁸. An additional study using the transgenic amyloid AD 5XFAD mouse model found that microglia depletion with PLX3397 reduced A β plaque deposition, as well as improved mouse performance on cognitive fear conditioning tasks¹⁴². Finally, in a model of AD-related tauopathy (TEKO), mice administered PLX3397 for 3.5 months were completely rescued from their known neurodegenerative phenotype¹⁴³.

There are several disadvantages of the PLX3397 compound that can be improved, such as its relatively low brain penetrance (5%) and the dual c-Kit/CSF1R inhibition (rather than specificity to CSF1R which is exclusively expressed on tissue resident macrophages). To address these limitations, structural modifications were made to

PLX3397 to generate an updated small molecule that is specific to CSF1R, more aggressively depletes brain microglia, and exhibits higher (20%) brain penetrance: PLX5622¹³⁹. Using a formulation of 1200mg/kg in mouse chow, PLX5622 has been shown to deplete >97% of brain microglia in as little as 5 days in adult mice¹³⁹. The use of this inhibitor in transgenic 5XFAD mice showed that complete depletion of microglia altered A β plaque pathology by reducing plaques in the brain parenchyma, but was later found to be at the cost of localizing plaques to the neurovasculature¹³⁹. Therefore, microglia in A β models, while thought to be negatively impacting the brain microenvironment, may be assisting by initially compacting plaques and protecting from further damage, as vascularization of plaques to the vasculature can cause increased risk of stroke and CAA¹⁴⁴.

The development of these small molecule inhibitors against CSF1R has also allowed for the exploration of microglia-neuron interactions in adult animals, which were thought to be minimally impactful. It was recently shown that microglia depletion enhances synaptic connectivity without altering intrinsic electrophysiological properties of excitatory and inhibitory neurons, as well as increased perineuronal net formation in the adult mouse visual cortex¹⁴⁵. While these results could be reversed when the CSF1R inhibitor was withdrawn (allowing for microglia repopulation), these findings propose that there is a negatively impactful role for microglia in synaptic plasticity under homeostatic conditions. Other studies have argued this finding, suggesting that partial microglia depletion with PLX5622 reduces dendritic spine density while increasing immature synaptic elements in CA3-CA1 hippocampal synapses in adult mice, as well as causes deficits in cognitive performance on the novel object recognition behavioral task¹⁴⁶. In aged mice, treatment with PLX5622 restored dendritic spine densities of hippocampal pyramidal cells¹⁴⁷. Furthermore, depleting brain microglia with PLX5622 in 10 month-old

transgenic 5XFAD mice (a later stage of disease for this model) was shown to rescue both neuronal and synaptic loss also observed in the hippocampus¹¹⁶. Together, these studies implicate a critical role for microglia in controlling neuronal health in both adulthood and pathological conditions. However, current studies are less specific about the neural circuitry and disease stages that microglia-neuron interactions are the most critical, as the beneficial outcomes would be to understand how these processes interact at an earlier time-point. Finally, it is still not well understood how genetic context modulates these interactions in promoting neurons or microglia to maintain resistance in aging as well as resilience to disease pathologies.

1.5 Neuronal circuits

1.5.1 Measuring synaptic dynamics on neuronal circuits

Synapses are the fundamental computational subunits of the brain, and the building blocks of neuronal circuits¹⁴⁸, which can vary in complexity based on brain region and mammalian organism¹⁴⁹. Neuronal circuits communicate in a cell-type and spatially specific manner to either receive connections from a discrete population (as observed in granule neurons in the cerebellum¹⁵⁰) or a diverse set of multiple connections onto a single cell (observed in pyramidal cells of the frontal cortex¹⁵¹). Synapses are tunable and plastic, which is critical for higher order learning and memory functions of an organism¹⁵², but strengthen upon repeated use, prioritizing certain connections to become stable¹⁵³.

Since synaptic structure often indicates the function and strength of a particular connection, many methods have been developed to measure these connections^{92,154,155}. One of the most studied aspects of synaptic structure are dendritic spines, which are membranous protrusions that form at the surface of an excitatory neuron when a

presynaptic input receives an incoming signal from a connecting cell¹⁵⁶ (**Figure 1.6**).

Functional studies have found that spines have biochemical and electrical properties, and their morphologies can dictate the amount of signal that is locally active at the spine head as well as diffusible through to the parent dendrite^{92,155,157,158}. Spine head morphologies are critical for determining the overall strength of local synaptic connections, with larger spines indicating stronger but less plastic connections, whereas smaller spines indicative of less stable, transient/plastic connections^{92,155}. Spine necks are thought to dictate how local synaptic signals are filtered to the parent dendrite. Spine neck widths are critical for dendritic compartmentalization, as wider necks allow for more signal to diffuse from the spine head to the parent dendrite whereas as neck width becomes smaller, less signal is able to pass¹⁵⁹. However, many imaging studies cannot capture the high resolution needed to obtain neck widths, and therefore the length of spine necks is often measured to determine functional compartmentalization. Spine neck lengths are inversely correlated to putative function, with longer necks having greater filtering capacity and therefore limiting diffusion to the parent dendrite, whereas shorter necks do not have as much filtering and allow for greater excitability to/from the parent dendrite and synapse¹⁶⁰.

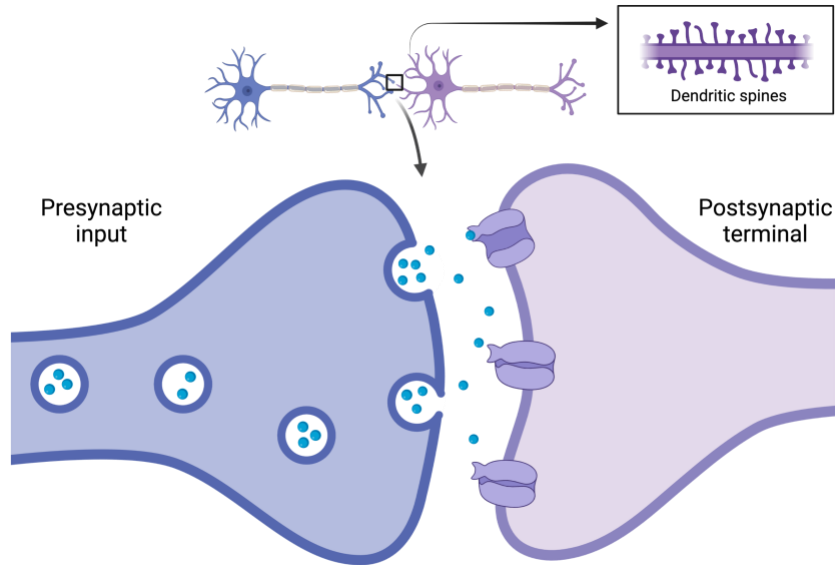


Figure 1.5: Illustration of the formation of a synaptic connection from excitatory neurons that create membranous protrusions across a dendritic branch, dendritic spines. Image created in BioRender.

The number (or density) of dendritic spines have become an accepted measure of synaptic strength of a neuronal population, as many labeling and imaging technologies have been developed that can quantitatively analyze this aspect of neuronal dendrites in a relatively high-throughput manner. This was established as early as the foundational experiments performed by Ramón y Cajal that used Golgi staining to uncover structural properties of dendritic spines¹⁶¹. Since these studies, many other techniques have been developed to label and analyze dendritic spines, including DiOlistic labeling that leverages a gene gun to deliver fluorescent dyes into a population of cells¹⁶². This approach is often faulted due to the random nature in the way the cells are labeled, so others have used approaches such as iontophoretic cell filling to manually label specific neurons^{163,164}. However, this process is still somewhat time consuming and does not achieve the study of specific neuronal circuitry. Furthermore, this approach is often used in nonhuman primate studies due to the lack of experimental flexibility and high amount of control these models often require¹⁶⁵. To address many of the limitations of traditional methods for structural analyses of neuronal dendrites, recombinant adenoassociated

viruses (AAVs) have been engineered to retrogradely express recombinase proteins (such as Cre and Flp), which can be combined with additional AAVs that drive expression of a particular fluorophore (usually EGFP or TdTomato) under a recombinase-specific promoter¹⁶⁶ (**Figure 1.7**). These techniques have been key to understanding the dynamics of neuronal projection pathways as well as uncovering density and morphologies of dendrites and dendritic spines¹⁶⁷. AAV-based neuronal circuit labeling was leveraged in this thesis to evaluate dendritic structure and spine dynamics across diverse genetic contexts, as well as determine circuit-specific effects of aging and neuropathologies on neuronal populations that are known to be vulnerable in either aging or AD.

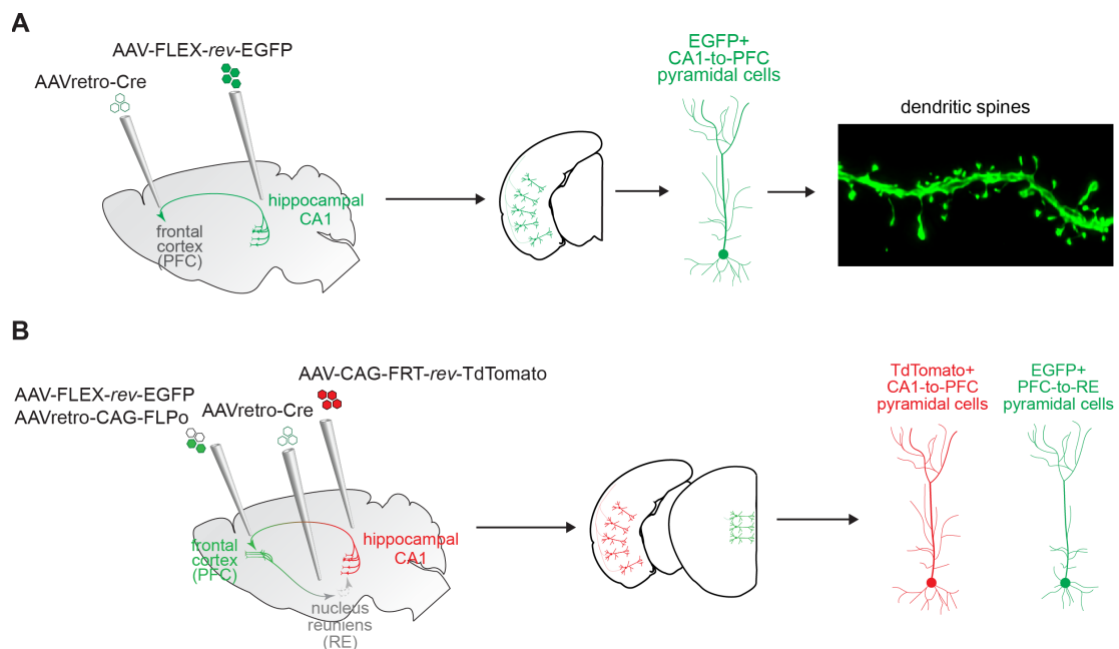


Figure 1.6: Examples of labeling techniques leveraged here to examine neuronal circuitry in aging and AD. (A) Single-circuit labeling using rAAVs to examine one population of neurons. (B) Dual-circuit labeling to simultaneously examine two neuronal circuits.

1.5.2 Neuronal circuit dysfunction in aging

Normal aging in the CNS is characterized by an overall loss of synapse number and plasticity in otherwise intact neuronal circuits¹⁶⁸. This is reflected by a slow and progressive loss in cognitive function and memory performance¹⁶⁹. However, neuronal cell death is virtually absent in the aged brain which is one of the differentiating factors in the process of normal aging versus AD^{170,171}. The specific brain regions and circuits affected during aging and AD are also different, with synaptic loss in the CA1 region of the hippocampus (HC) and entorhinal cortex (EC) more specific to AD, whereas normal aging is hallmarked by synaptic loss in the prefrontal cortex (PFC)⁷². The reports of degree to which this synaptic loss occurs during aging and AD is variable, which could be due to a number of biological and technical factors^{163,168,172,173}. Some suggest synaptic and cognitive aging is governed by genetic factors, and could determine who is defined as successful versus unsuccessful ager^{72,168,174}. However, many other conditions such as environmental influences and biological sex may define aging trajectories, which calls for the use of more refined model systems and tools to investigate these mechanisms.

The primary experimental platforms (outside of human studies) that have been used to study the aging synapse have been in model mammalian systems from non-human primates (NHPs) and rodents (rats and mice)¹⁷⁵. Having the highest degree of translatability to human brain structure and neuronal circuitry, NHPs are the most traditionally used system that has identified age-related synaptic loss in the frontal cortex that corresponds to cognitive decline^{165,176}. However, NHPs are a challenging experimental system, as many have incredibly long lifespans (>30 years), they produce small numbers of offspring during each gestational period, require complex housing systems, and have low amenability to genetic-based experimentation. To address these issues, studies in mice have demonstrated age-related synaptic loss in specific brain

regions such as the frontal cortex^{163,170,173}. Aged mice have also generated significant amounts of data that study synaptic loss during aging and in age-related diseases^{52,177}, proposing them as a beneficial model system to understand the mechanisms that discriminate successful from unsuccessful aging. Using tools available including diverse mouse strain resources, the genetic components that drive cognitive and synaptic aging in known vulnerable (and resistant) neuronal circuits were leveraged in this thesis.

1.5.3 Neuronal circuit dysfunction in AD

Compared to normal cognitive aging, individuals affected by AD exhibit decline across cognitive domains including learning and memory, and executive functioning⁹. Imaging studies have shown the accumulation of AD-defining neuropathologies are often associated with progressive yet selective patterns of synaptic disruption that emerge in EC and HC circuits before becoming widespread across the frontal cortex and parietal association areas^{72,178}. Disruptions in neuronal and synaptic dynamics in AD are thought to occur well before neurodegeneration and cognitive deficits are observed¹⁷⁹. However, AD-related synaptic loss is not homogenous across the brain, but rather affects brain regions and circuits dependent on clinical and pathological stages of disease. Additionally, the disruption of neural networks and circuits in AD is distinct from those affected during normal cognitive aging (as discussed in the sections prior) with AD exhibiting specific susceptibility in HC circuits⁷².

The primary theory of circuit disruption in AD is that it follows the spread of related neuropathologies (**Figure 1.8**). One of the first AD symptoms is episodic memory loss which corresponds to the observed early spread of neuropathological tau tangles and A β plaques¹⁸⁰. The cognitive deficits that parallel pathological spread translate to small animal studies that lesioned the EC in rats and observed impaired performance on

episodic memory behavioral tasks as well as impairment on other learning processes, reflecting symptoms observed early on in AD patients^{181,182}. A foundational study in humans examined postmortem brain tissue from AD patients and found that A β plaque spread originates in the neocortex, in regions responsible for general cognitive function, spreading to learning and memory hubs such as the HC in early to mid-stages of disease, and in final stages spreading to motor and sensory neural structures¹⁸³. However, while the stages of pathological propagation in AD have been well characterized for the past several decades, it is still unclear how this deposition occurs and why specific neuronal circuits and networks become vulnerable to synaptic dysfunction and eventual loss as the disease progresses, or even if the relationship between pathology and synaptic dysfunction is as straightforward as initially proposed.

At a finer resolution, neuronal circuit dysfunction in AD is thought to occur because A β oligomers accumulate at synapses which causes an imbalance of excitatory and inhibitory signals, eventually impairing measures of neuron function such as LTP^{179,184–186}. The areas of observed A β plaque accumulation are often where the greatest degree of excitatory neuron synaptic loss is observed in later disease stages^{187,188}. During earlier disease stages however, excitatory neurons play a compensatory role and are thought to increase their activity through the mechanism of A β plaques causing hyperexcitability¹⁸⁹. These processes have been observed both *in vitro* through experiments using cultured cortical neurons, as well as in AD mouse models where hyperactive clusters of neurons have been identified within close proximity to A β plaques^{184,190}. The increased activity in excitatory neurons may provide explanation for the susceptibility of AD patients to epileptic seizures, which are also seen in AD mouse models^{191,192}.

A final component to synaptic dysfunction in AD is the role of microglia and their effects on vulnerable neuronal circuits. Microglia are thought to drive circuit dysfunction

in AD, which is largely supported by their known role in regulating synaptic connectivity during development and in the healthy, adult brain (as discussed previously)^{129,193}, and observations that microglia depletion in AD mouse models reduces late-stage hippocampal synapse loss^{116,147}. Furthermore, the selective vulnerability of hippocampal and EC circuits occurs alongside robust neuroinflammation¹²⁷, which posits that microglia activation in response to A β plaque deposition is possibly a causal event driving synaptic dysfunction and neuronal circuit disruption¹⁹⁴. However, the precise relationship between A β deposition, microglia activation, and neuronal synapse disruption in specific AD-vulnerable circuits is still to be determined. Furthermore, the degree to which genetic context drives resilience mechanisms that govern microglia-synaptic disruption is what will be explored further here in this thesis.

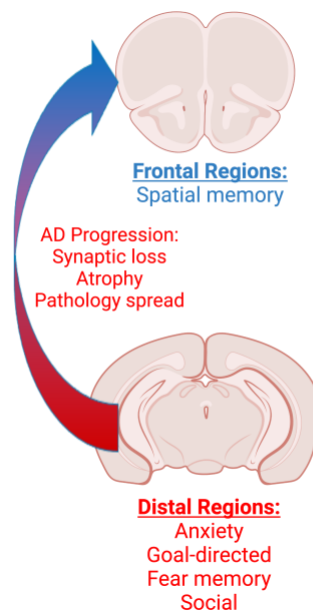


Figure 1.7: Neuronal circuit dysfunction in AD follows spread of amyloid and tau pathology, beginning in hippocampal and entorhinal cortical regions, and spreading to frontal areas. Image created in BioRender.

1.6 Project rationale

This thesis explores the mechanisms that drive susceptibility, resistance, and resilience to aging and AD-related cognitive dysfunction through the study of novel genetically diverse mouse models. The first results chapter (Chapter 2) leverages the population of genetically diverse AD-BXD mice to develop a prediction model that proposes diversity in one's genetic background (combined with A β pathological insults) causes interactions between microglia and neurons that leads to variability in AD cognitive outcomes. This model developed the hypothesis that resistance to aging and resilience to AD are governed by genetic context by influencing the degeneration of vulnerable neuronal circuits, and in AD microglia are critical to this degeneration. The latter part of this thesis (Chapters 3 & 4) specifically focuses on two genetically distinct mouse strains that exhibit diverse performance on cognitive tasks: the inbred laboratory B6 (cognitively susceptible) and wild-derived inbred PWK (cognitively resilient). In this thesis I leveraged B6 and PWK mice to investigate how PFC and HC neuronal circuits degenerate during aging and AD, and how the depletion of microglia impacts hippocampal-specific degeneration. Together, work from this thesis leverages diverse genetic mouse models to support a role for microglia in causing disease susceptibility, and nominates the PWK mouse strain as a model to study further for resistance/resilience mechanisms to aging and AD.

Chapter 2: Identifying the molecular systems that influence cognitive resilience to Alzheimer's disease in genetically diverse mice¹

¹**Heuer SE**, Neuner SM, Hadad N, O'Connell KMS, Williams RW, Philip VM, Gaiteri C, Kaczorowski, CC. *Learning and Memory*. 2020;27(9):355-371. PMID: 32817302.
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2.1 Introduction

Alzheimer's disease (AD) dementia is a common neurodegenerative disorder defined by extracellular amyloid plaques and intracellular neurofibrillary tangles, and characterized by progressive memory decline¹⁹⁵. Many of the tested therapies for AD have focused on targeting and clearing pathological beta-amyloid ($A\beta$), which has been extensively researched as an initiating factor that leads to neurodegeneration and cognitive impairment^{196,197}. To date, therapeutics to delay or reverse the progression of AD-related cognitive decline have been disappointing, despite significantly removing $A\beta$ burden^{198,199}. These drug failures, and investigations into the origin of AD more broadly, are both impacted by the divergence between the clinical (cognitive decline) and pathological (amyloidosis and hyperphosphorylated tau) presentation of the disease, with some cognitively intact elderly individuals exhibiting significant amyloidosis consistent with new diagnostic criteria for AD^{200–202}. Even individuals with well-defined familial AD (fAD) mutations in amyloid precursor protein (*APP*) or presenilin 1 (*PSEN1*) genes can maintain cognitive function well beyond their sixth decade, despite robust amyloid and tau pathological burden, identifying individuals that are resilient to pathology-induced cognitive decline^{22,25,203,204}. In light of this disconnect between AD pathology and cognitive symptoms, there is a critical need to understand how genetic background can modify the impact of amyloid on variation in AD cognitive decline.

Recent advances in genomic techniques have enabled detailed investigation of cognitive decline using genome-wide transcriptional profiling from postmortem brains of demented and non-demented control individuals^{205–208}. Although these studies have identified some of the processes associated with cognitive decline, such as increased brain inflammation and neurodegenerative pathways, there is still an incomplete understanding of the factors that directly drive cognitive outcomes in dementia patients. One recent study employed an integrative approach which combined postmortem gene

co-expression, measures of cognitive decline, and measures of pathology to generate a Bayesian conditional-dependency network that identified several modules predicted to directly influence cognitive decline without pathology⁷⁸. This was a key development in understanding the complex etiology of AD-related cognitive decline in that rather than identifying correlational relations, this study employed Bayesian directed modeling that incorporated molecular networks, pathological, and cognitive measures to propose a candidate biological network that directly influenced cognitive decline without the direct influence of pathology. However, the reliance on postmortem tissues limits our ability to define early molecular changes that may drive onset of dementia, especially since many of these processes start long before the onset of cognitive symptoms^{209–211}.

To identify novel genetic factors and molecular systems that are responsible for resilience to cognitive symptoms of AD longitudinally and under controlled environmental conditions, we systematically examined the effect of fAD mutations on cognitive traits in a genetically diverse panel of mice; the AD-BXDs⁴⁸. The AD-BXD genetically diverse mouse model of AD incorporates the commonly used 5XFAD transgene across a number of diverse, yet inbred, mouse strains from the BXD genetic reference panel⁴⁸. We found that the AD-BXDs exhibited variation in cognitive decline similar to that observed in human fAD populations²². This variation was sensitive to genetic risk factors associated with human sporadic AD⁴⁸, suggesting this is a translationally relevant model of late-onset AD (LOAD).

The goal of this study is to build on our previous findings to further prioritize the molecular systems which likely account for the wide range of cognitive outcomes using a population of identical individuals with and without AD mutations. We took a systems biology approach combining genetic, transcriptomic, and behavioral data from a large population of normal aging (Ntg-BXD) and AD (AD-BXD) cases (**Figure 2.1**).

Specifically, we inferred gene co-expression modules from hippocampus transcriptome

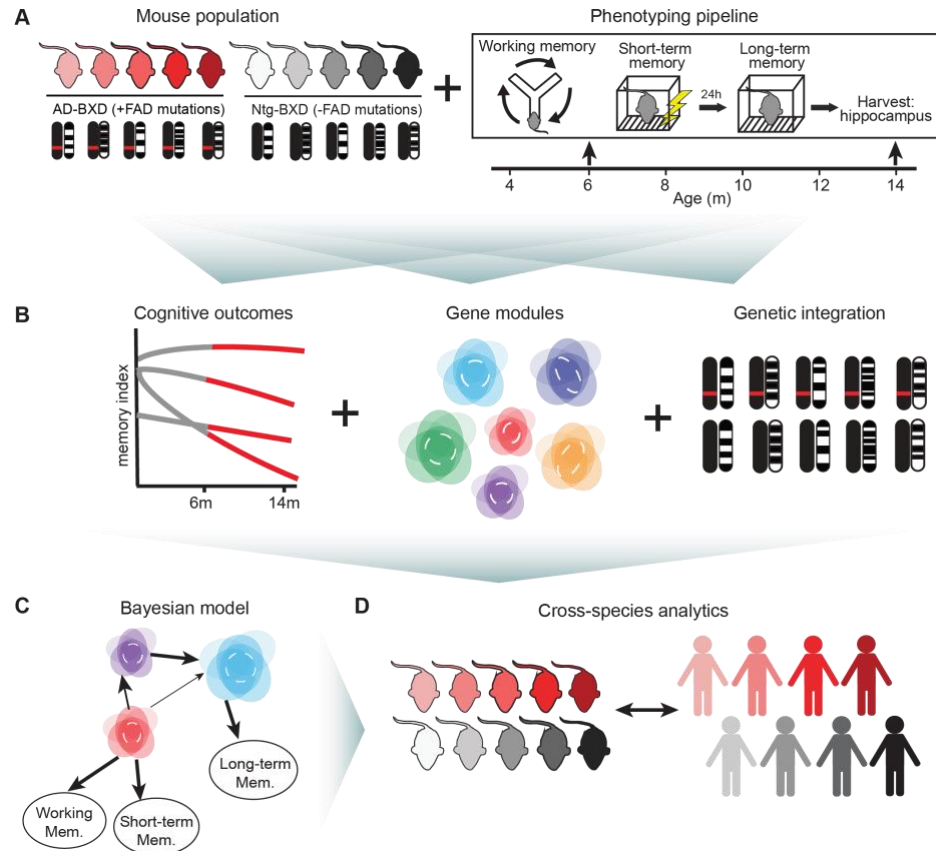


Figure 2.1: Genetically diverse mice can be leveraged to understand the relevant molecular networks that underlie individual differences in cognitive outcomes. Schematic of SpeakEasy module formation and relation to cognitive outcomes, adapted from Mostafavi et al., 2018. A) Multiple strains of genetically diverse mice that are carriers (AD-BXD) or non-carriers (Ntg-BXDs) for the 5XFAD transgene (fAD mutations) are combined to generate a population with genetic diversity similar to what is observed in humans. These strains make up a genetic reference panel, allowing for the incorporation of genetic information into further analyses. Each mouse is brought through a cognitive phenotyping pipeline (described in Neuner et al., 2019a), taking measures for working memory, short-term memory, and long-term memory at 6m or 14m of age, and harvest of hippocampal tissue for downstream analyses. B) Measures of the three cognitive domains taken during the phenotyping pipeline are assessed from AD-BXD and Ntg-BXD mice at 6m and 14m of age (left), while gene co-expression modules are separately formed from the same mice using SpeakEasy consensus clustering (middle). Additionally, genetic information is obtained from each strain for downstream genetic mapping and integration into the module-trait network (right). C) Gene expression modules are related to cognitive traits using Spearman correlations, and modules significantly (adj. $p < 0.05$) correlated to at least one cognitive measure are selected for downstream analysis. These cognitively relevant modules are integrated with cognitive measures, and anchored by BXD genetics to form a module-trait Bayesian directed network, and modules directly upstream of cognitive traits are prioritized for further characterization. D) Cognitively relevant modules are compared with human brain modules to investigate gene-gene conservation between mouse and human.

data of a genetically diverse mouse population from young [6 months old (6m)] and aged [14 months old (14m)] individuals. Each of these modules represents the activity of one or more molecular systems, and was identified in a purely data-driven manner, blind to genotype or behavioral outcomes. Then, we identified which modules and molecular systems were associated with age, amyloid mutations, and cognitive outcomes. Genetic mapping of module eigengene expression as quantitative traits identified genetic regions that regulated each module. We then used Bayesian conditional dependency modeling²¹² to eliminate redundant associations and predict the subset of modules driving individual differences in cognition across the population, and calculated if these relations were dependent on the presence of amyloid-driving mutations, and if module-trait relations were strengthened by the addition of cortical amyloid burden into our statistical model. Finally, we tested if the prioritized molecular networks from this study are translationally relevant by testing the overlap between mouse and human dementia networks⁷⁶⁻⁷⁸. This approach nominates robust molecular systems that consistently relate to individual differences in cognition in an aging and AD populations, and evaluates the effect of these systems on cognitive outcomes. Furthermore, additional modeling that incorporated cortical A β 1-42 ELISA suggests an indirect relation of AD pathological burden on cognitive outcomes in promoting differences in molecular network activity, and highlights the utility of mouse models for discovering several molecular systems to monitor for resilience and manipulate them in future validation studies to improve AD cognitive outcomes.

2.2 Results

2.2.1 Defined molecular systems of the hippocampus in AD-BXD and Ntg-BXD mice

In order to identify the molecular systems associated with cognitive outcomes across the population of both AD and non-AD mouse strains, we first obtained RNA sequencing (RNAseq) data from the hippocampus of AD-BXD and Ntg-BXD mice at either 6m or 14m of age ($n = 294$)^{48,59}. Biological replicates from the strain, sex, and genotype groups were averaged together, generating a total of 156 unique group-averaged measures. With these data, we then performed unsupervised consensus clustering and identified 28 discrete transcriptional modules of >29 genes, ranging in membership size from 42 to 1419 transcripts. We commonly refer to these modules as gene sets, but recognize that in this context we more formally mean sets of mRNAs with one-to-one relations to genes. We chose the SpeakEasy consensus clustering algorithm over other clustering algorithms, such as Weighted Gene Co-expression Network Analysis (WGCNA), because it is less parameter-driven and carries a higher degree of run-to-run stability for transcriptome network conservation^{70,78}. To gain a basic understanding of the biological processes and cell-types enriched in each module, we performed Gene Ontology (GO) biological process enrichment on each module, as well as cell-type enrichment using a well-established and mineable RNAseq dataset from mouse cortex²¹³. The major molecular functions and module-module relations of these gene sets are highlighted in **Figure 2.2**. Modules that were clustered together based on covariation in expression are also similarly characterized based on cell-type and biological function, without having overlapping members since each module was formed using discrete gene assignment. These included a group of modules enriched for genes expressed in neurons, as well as synaptic organization and intracellular transport GO terms, a large grouping of astrocyte modules enriched for RNA processing, cell movement, and chromatin remodeling GO

terms, a mixed endothelial cell and astrocyte grouping of modules enriched for metabolism GO terms, and a microglia module group enriched for immune function GO terms (**Figure 2.2**). The annotation of these covarying molecular systems identifies the major cell-types and biological pathways present in the hippocampus of our population, a subset of which may change as a function of aging and/or disease processes.

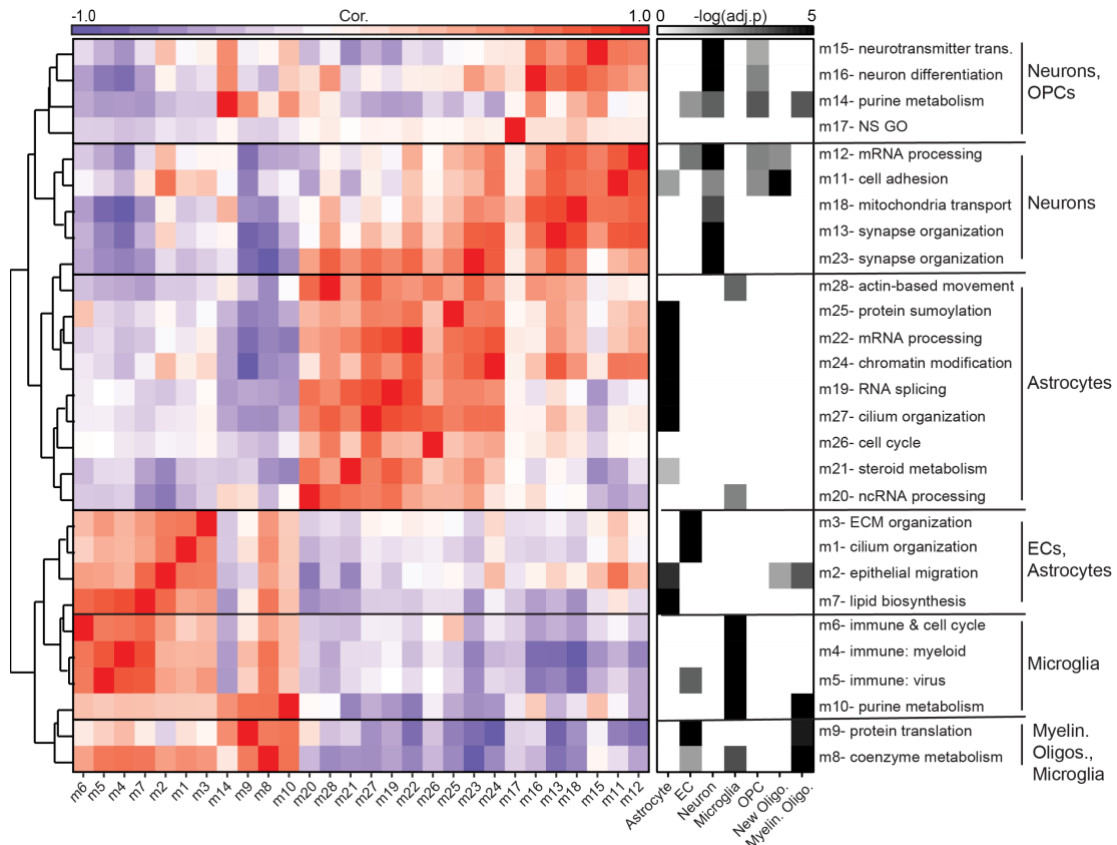


Figure 2.2: AD-BXD and Ntg-BXD have variable transcriptional networks across aging and Alzheimer's individuals. SpeakEasy consensus clustering identified 31 discrete transcriptional modules from hippocampal RNA sequencing of Ntg/AD-BXD mice ($n = 26$ strains). Modules containing <30 genes were excluded ($n = 3$ modules). Average module expression per sample was derived using average standardized gene expression of module members. Modules were correlated to each other (Pearson) and hierarchically clustered. Cell-type enrichment was performed on the genes within each module using the Zhang *et al.*, 2014 dataset to obtain an idea of represented cell-types for each module. Cell-type enrichments were performed using hypergeometric tests to identify significant relationships ($\text{adj. } p < 0.05$) for astrocytes, endothelial cells (EC), neurons, microglia, oligodendrocyte precursor cells (OPC), new oligodendrocytes (New Oligo.), and myelinating oligodendrocytes (Myelin. Oligo.). GO enrichment was performed on the genes in each module, and the GO term most significantly enriched within a module is denoted (NS GO = no significant GO enrichment).

2.2.2 Prioritizing molecular systems related to cognitive outcomes highlights immune and metabolic networks

The co-expression analysis described above defined the basic molecular systems that can be robustly identified and evaluated in mouse hippocampi. Importantly, these mRNA sets are formed without any reference to genotype or phenotype. Therefore, we next considered how the average expression of each co-expression module was related to sex, age, and fAD mutation carrier status (**Figures 2.3, 2.4, 2.5**), and cognitive status (**Figure 2.2**). To do this, we first generated a summary statistic for each module by calculating the average standardized expression of the gene members for each module. Next, we compared each module summary statistic with strain-averaged performance on cognitive tasks using Spearman correlation analysis. These tasks spanned multiple cognitive domains and included working memory using spontaneous alternation on the Y-maze and acquisition and recall of contextual fear conditioning at both 6 and 14 months of age. Spontaneous alternation relies on a rodent's innate tendency to explore novel environments while avoiding familiar environments and assesses hippocampal- and cortical- dependent spatial working memory. This task was initially chosen due to the lack of need for training, reward, or punishment, and the ability to test mice on this task longitudinally^{40,214,215}. Contextual fear conditioning enables the assessment of both hippocampal-dependent short-term memory during the acquisition phase in which mice are administered 4 mild foot shocks^{64,216}, as well as hippocampal-dependent spatial orientation, recall, and long-term memory during the testing phase in which mice are placed back into the training chamber in the absence of foot shocks 24 hours after contextual fear conditioning training^{64,65}.

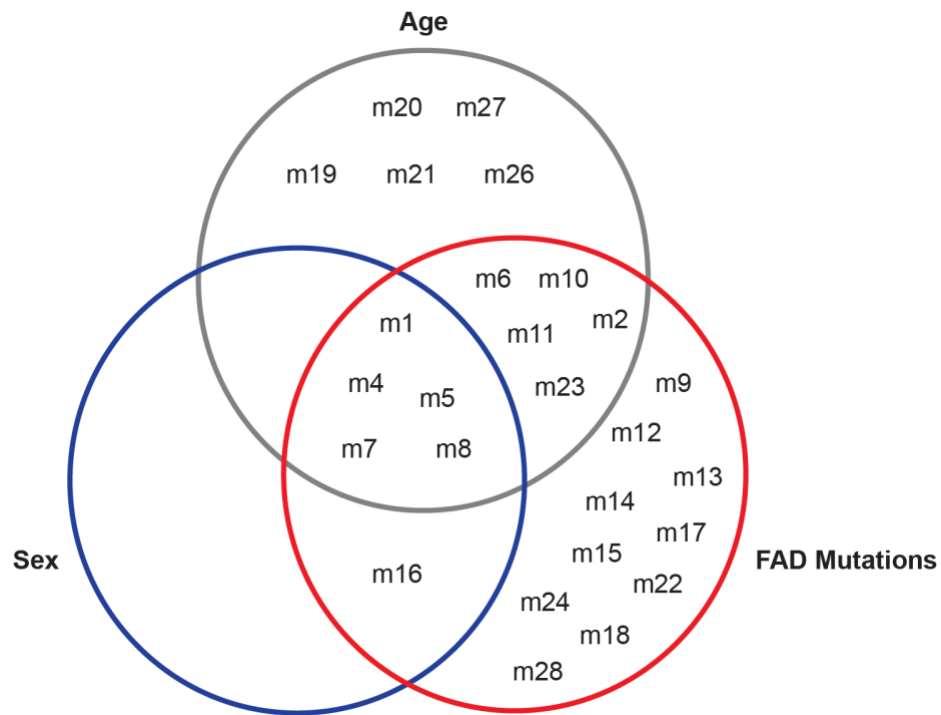


Figure 2.3: ANOVA identifies covariates that influence module expression. Venn diagram illustrates how the expression of each module was significantly influenced by fAD mutations/genotype (red), age (grey), or sex (blue). Significant relationships determined for each covariate using three-way Type 2 ANOVA (Benjamini Hochberg adj. $p < 0.05$). Two modules (m3, m25) were not significantly affected by any of the three factors after adjusted p-value correction and are not depicted above.

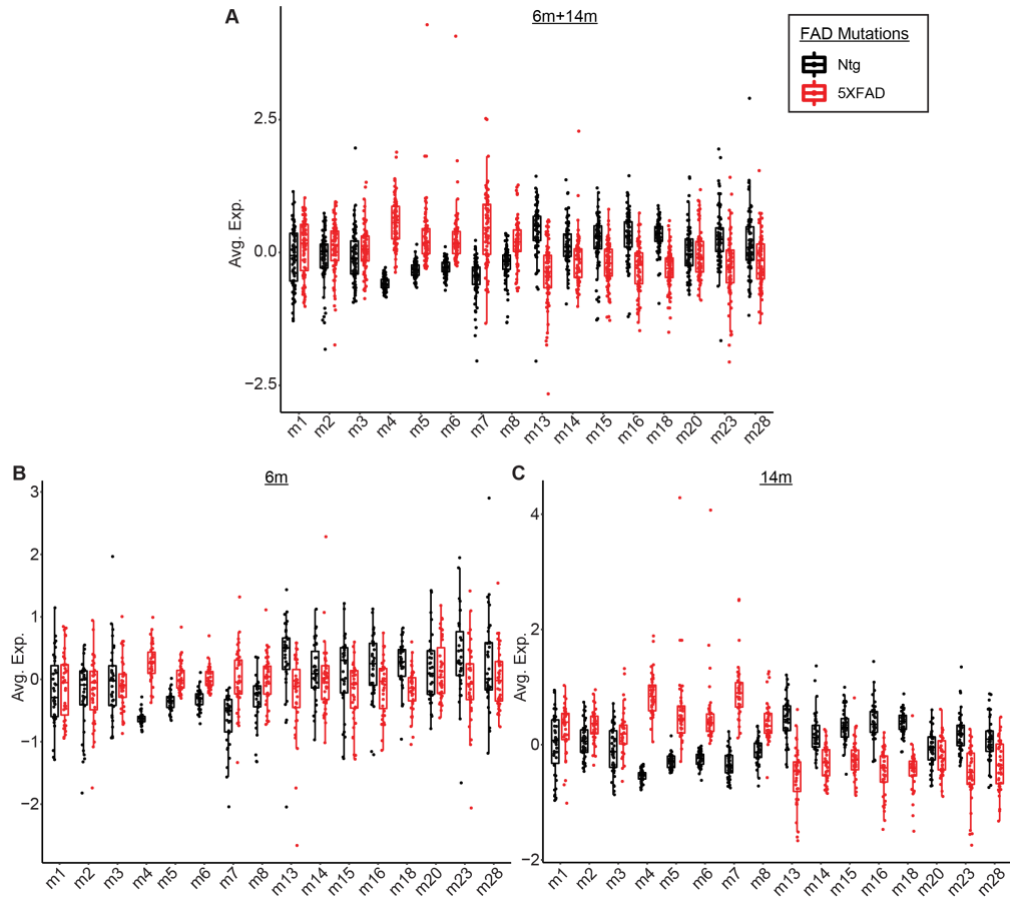


Figure 2.4: Variation of module expression as a function of fAD mutations. Module expression for each of the 16 cognitively relevant modules was visualized within 5XFAD (red) and Ntg (black) BXDs for all individuals (A), and separated 6m (B) and 14m (C) populations.

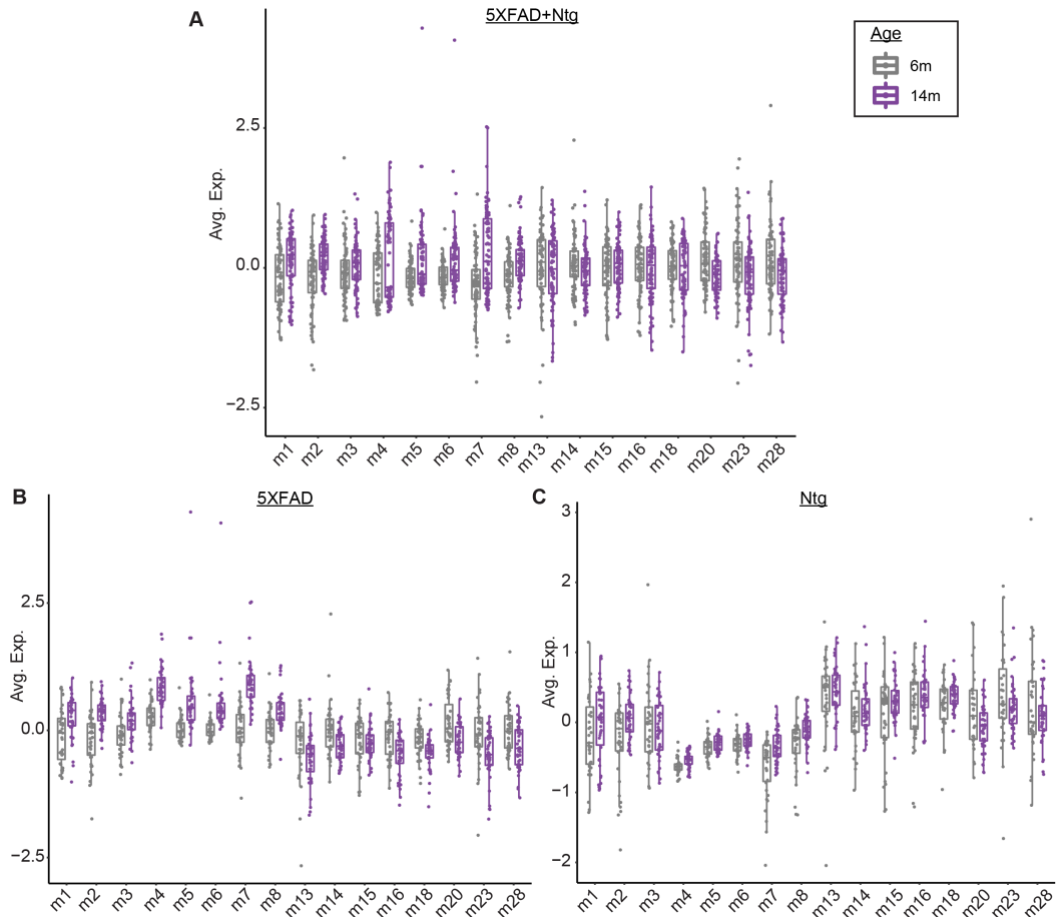


Figure 2.5: Variation of module expression as a function of age. Module expression for each of the 16 cognitively relevant modules was visualized within 6m (grey) and 14m (purple) BXDs for all individuals (A), and separated 5XFAD (B) and Ntg (C) populations.

We identified 16 modules that significantly correlated with at least one cognitive outcome (**Figure 2.6A**; Spearman, adj. $p < 0.05$). There were 6 modules (5 negative, 1 positive) significantly correlated with percent successful spontaneous alternations (%SA) on the Y-maze working memory task. The modules negatively correlated with %SA on the Y-maze working memory task (m2, m4, m6, m7, m8) contain genes highly expressed in microglia, and enriched for GO biological processes including cell migration, immune function, and lipid biosynthesis processes. The only module positively correlated with %SA on Y-maze (m20) contained members that are also expressed in microglia and

were enriched for non-coding RNA processing GO terms. There were 16 modules (8 negative, 8 positive) that significantly correlated with contextual fear acquisition (CFA) slope (see *Methods*). The negatively correlated modules contained genes expressed in astrocytes and microglia and were primarily enriched for GO terms associated with immune function (m4, m5, m6) as well as cell migration (m1, m2, m3) and lipid biosynthesis and metabolism (m7, m8). The positively correlated modules contained genes expressed primarily in neurons and were enriched for GO processes associated with synaptic signaling and organization (m13, m15, m16, m23, m28), mitochondrial transport (m18), and RNA processing and metabolism (m14, m20). Finally, there were 6 modules (5 negative, 1 positive) significantly correlated with contextual fear memory (CFM). The negatively correlated modules contained genes expressed in microglia and astrocytes and were enriched for migration (m2), immune (m4, m5, m6) and lipid biosynthesis (m7) processes. The positively correlated module (m14) was enriched for genes associated with purine metabolism processes and contained genes expressed in multiple cell-types including endothelial cells, neurons, microglia, and myelinating oligodendrocytes. One thing to note is that we identified four modules that were significantly correlated with all three cognitive outcomes (m2, m4, m6, m7), suggesting perturbation of these modules would influence all three cognitive domains, instead of being-domain specific processes. Together, results from these analyses suggest that increased number of transcripts associated with antiviral and innate immune pathways in microglia, as well as lipid biosynthesis pathways in astrocytes associate with lower cognitive performance, while synaptic signaling and purine metabolism pathways that are enriched in neuron and oligodendrocyte precursor cells are associated with higher cognitive performance (across all ages and genotypes), highlighting these biological networks as resilience candidates.

In addition to identifying significant module-trait relations, we also tested the extent to which significantly correlated modules explained variance in cognitive outcomes across the population. Initially, we calculated heritability of each trait to determine the amount of variance genetic background (or BXD strain) explained for each cognitive outcome. Similar to our previous findings⁴⁸, the majority of the variance in cognitive outcomes could be explained by genetic background, with 73% heritability for %SA on Y-maze working memory, 70% heritability for CFA slope, and 89% heritability for CFM. Using three-way ANOVA we separately calculated the amount of variance in each cognitive outcome that could be explained by binary-coded variables such as age, sex and presence of fAD mutations (**Table 2.1**). Age was the only variable that significantly affected %SA on Y-maze working memory, explaining 8.2% of the variance in %SA. Additionally, age was the only variable explaining significant variance for CFM, explaining 12.7% of the variance in %freezing on this task. Finally, age and fAD mutation carrier status explained significant variance for CFA slope, with 4.6% explained by age, and 19.9% explained by fAD mutation carrier status.

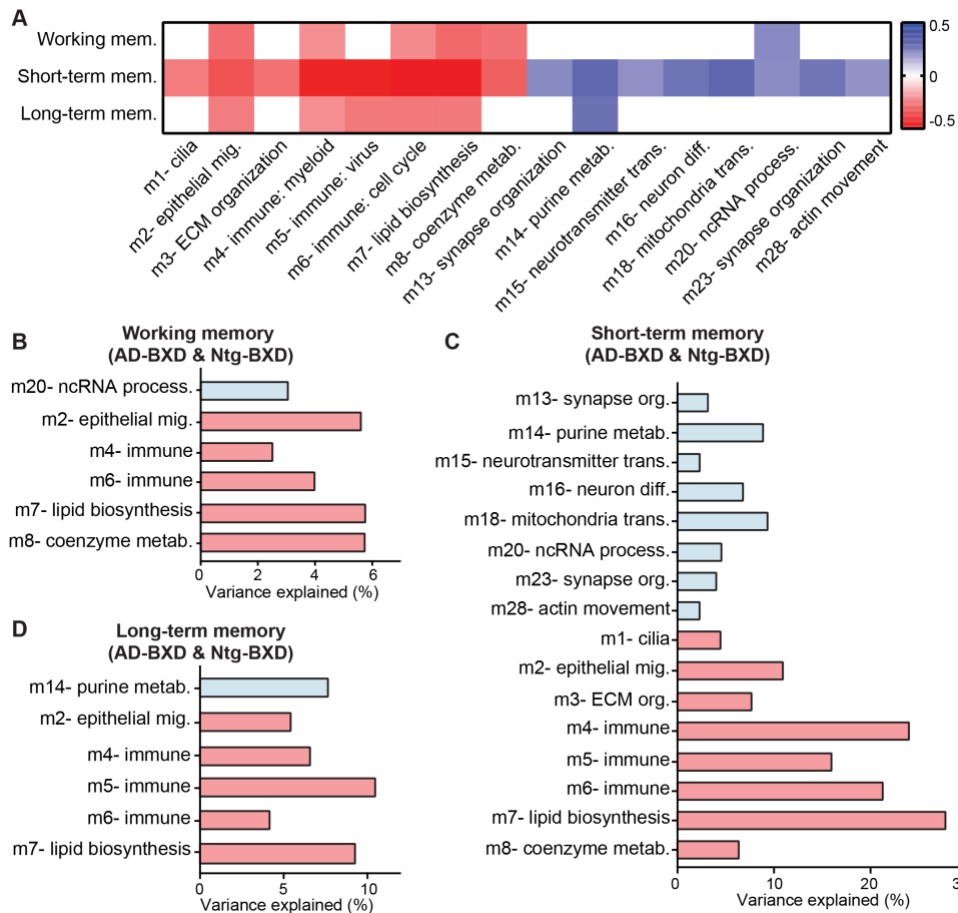


Figure 2.6: Correlation analysis reveals a select sixteen modules related to cognitive outcomes. A) Average standardized module expression was correlated to cognitive traits to identify modules that may significantly modify cognitive function. Modules significantly (Spearman, adj. $p < 0.05$) correlated to measures of working, short-term, and long-term memory are highlighted above. B-D) The proportion of variance in measures for working memory (B), short-term memory (C), and long-term memory (D) explained by individual module average expression. Bars are colored based on directionality of correlation to cognitive measure, with positive correlations in blue and negative correlations in red. All modules are denoted by the most significantly enriched GO term (NS GO = no significant GO enrichment).

Table 2.1: Three-way ANOVA reveals binary-coded variables that influence cognitive outcomes.

Trait	Genotype		Age		Sex	
	p-value	%variance	p-value	%variance	p-value	%variance
Y-maze (%SA)	0.35	0.52	0.00031	8.2	0.48	0.3
CFA Slope	2.72E-9	19.9	0.0028	4.6	0.36	0.42
CFM (%freezing)	0.13	1.3	4.98E-6	12.7	0.24	0.78

Using linear regression modeling, the variance of each cognitive outcome explained by module expression was calculated and employed as a proxy for effect size of module expression on cognitive outcomes. We focused only on module-trait relationships that reached statistical significance in our Spearman correlation analysis, and performed individual linear regressions for each module-trait pair. The modules that explained the most variance for working memory (%SA) outcomes were the epithelial migration module (m2, 5.6%), lipid biosynthesis module (m7, 5.8%) and coenzyme metabolism module (m8, 5.7%) (**Figure 2.6B**). Variance in CFA short-term memory outcomes was explained more strongly and by a larger number of modules than %SA, including immune modules (m4, 24.0%; m5, 16.0%, m6, 21.3%), and the lipid biosynthesis module (m7, 27.8%) (**Figure 2.6C**). The modules that explained the greatest amount of variance for CFM long-term memory outcomes included the immune: virus module (m5, 10.5%), lipid biosynthesis module (m7, 9.3%), and purine metabolism module (m14, 7.7%) (**Figure 2.6D**). While these module-trait correlations can prioritize molecular systems in disease, the causal nature of these relationships is unclear. Therefore, we proceeded to integrate genetic priors and conditional dependences to further assess the robustness of the top findings from these initial results.

2.2.3 Genetic network analysis supports a role for lipid biosynthesis and viral immune enriched molecular networks in cognitive resilience

As the effect of a molecular system on a phenotype may be conditional on activation of other molecular systems, we tested for such relations in the module-trait associations we identified. In this analysis, we simultaneously utilized module expression for the 16 cognitive relevant modules, cognitive measures, as well as information about genomic regions involved in regulating module expression. Chromosomal regions that regulate module expression were identified using genetic mapping in which module eigengenes

were used as quantitative traits. According to the central dogma of expression genetics^{217,218}, DNA variants influence RNA expression, which then modulates downstream behavioral phenotypes such as cognitive outcomes²¹⁹. As such these chromosomal regions were used as causal anchor points in our Bayesian network analysis to attempt to identify which molecular networks (and chromosomal regions) were causally associated with cognitive outcomes. The modules predicted to directly influence cognitive outcomes were m7 (astrocytes, lipid biosynthesis) for Y-maze working memory and CFA short-term memory and m5 (microglia, immune: virus) for CFM long-term memory (**Figure 2.7**).

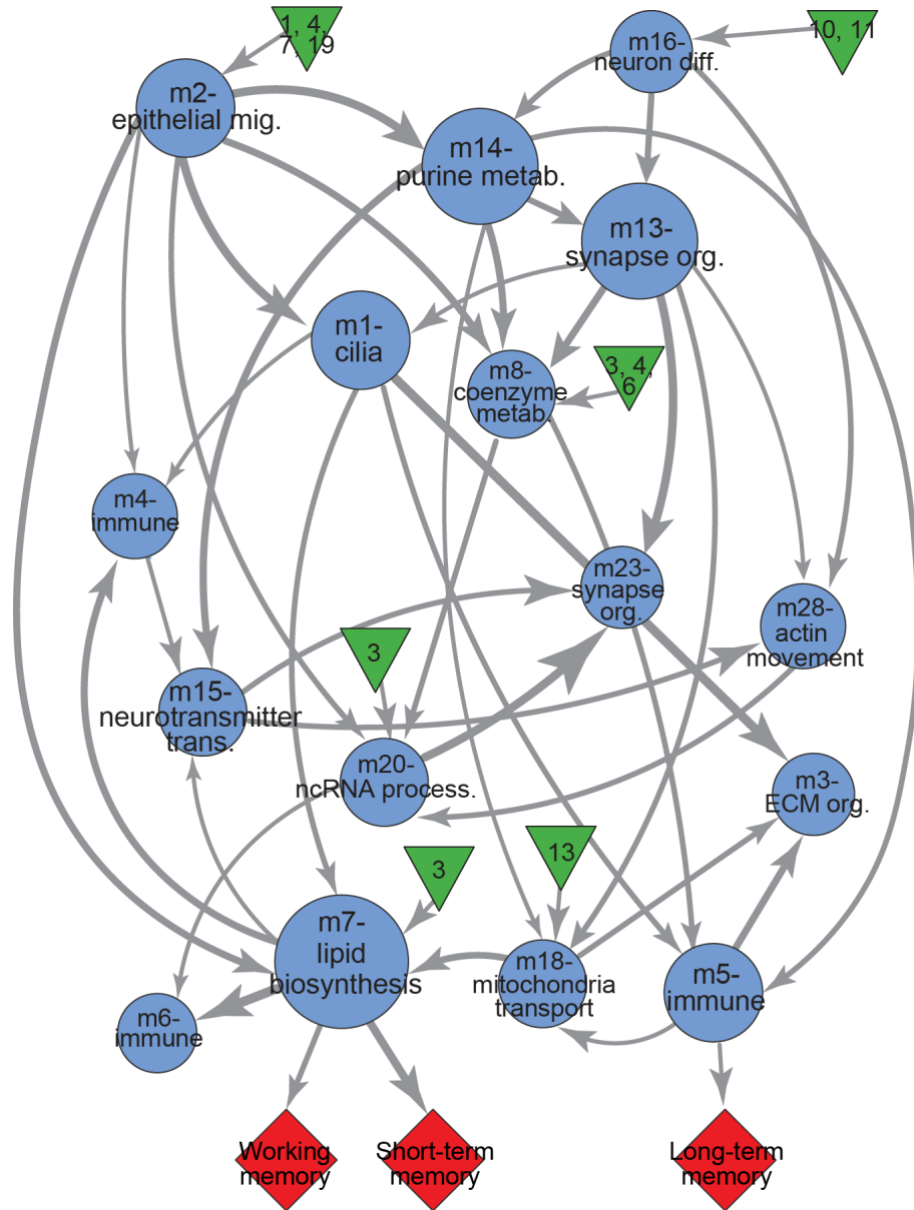


Figure 2.7: Bayesian conditional dependency modeling of cognitively relevant modules and traits defines causal module-trait relationships. Genetics (green triangles), module expression (blue circles, denoted by most significantly enriched GO term), and measures for each cognitive domain (red diamonds) were combined into a single module-trait network using Bayesian conditional dependency modeling. The genomic priors used in this analysis were the identified genetic regions associated with module eigengenes (PC1), and the chromosome(s) that each region mapped to is denoted inside each triangle. The thickness of the arrows represents the number of times a module-module or module-trait relationship was detected.

Table 2.2: Top Five IPA enriched canonical pathways for module 7 (m7, lipid biosynthesis).

IPA Canonical Pathway	P-value	Genes
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	3.89×10^{-6}	<i>Cebpg, Fgf2, Fzd9, Il17ra, Il33, Irak2, Irak3, Nfkbie, Plce1, Rap1b, Rhoa, Ripk1, Stat3, Tcf7l1</i>
Superpathway of Cholesterol Biosynthesis	8.13×10^{-6}	<i>Dhcr24, Dhcr7, Hmgcs1, Hsd17b7, Idi1</i>
Interleukin-1 (IL-1) Signaling	3.89×10^{-5}	<i>Gna12, Gna13, Gng12, Irak2, Irak3, Nfkb2, Nfkbie</i>
Actin Cytoskeleton Signaling	8.32×10^{-5}	<i>Cyfp1, Fgd3, Fgf2, Gna12, Gna13, Gng12, Gsn, Rap1b, Rhoa, Wasf2</i>
Regulation of the Epithelial-Mesenchymal Transition Pathway	1.58×10^{-4}	<i>Fgf2, Fgfr1, Fzd9, Hgf, Nfkb2, Rap1b, Rhoa, Stat3, Tcf7l1</i>

The module directly upstream of Y-maze working memory and CFA short-term memory, m7, also explained the most variance in these cognitive measures (**Figure 2.6B-C**). Module m7 contained 243 gene members, making it smaller in size in comparison to the three major microglia modules with 1088 members in m4, 848 members in m5, and 681 members in m6. This module was most significantly enriched for GO terms related to lipid biosynthesis and for genes that are highly expressed in astrocytes (**Figure 2.2**). Ingenuity Pathway Analysis (IPA) identified multiple molecular systems enriched in this gene set consistent with astrocyte activity including Interleukin-1 (IL-1) signaling, cholesterol biosynthesis and actin cytoskeleton signaling (**Table 2.2**). Specifically, the m7 genes enriched in the IL-1 signaling pathway were *Irak2* and *Irak3*, part of the IRAK family of proteins that are involved in direct downstream signaling of the IL-1 receptor, as well as *Rhoa*, which is important to actin cytoskeleton signaling.

Table 2.3: Top Five IPA enriched canonical pathways for module 5 (m5, immune: virus).

IPA Canonical Pathway	P-value	Genes
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	3.98×10^{-12}	<i>C3, Ccl5, Cntf, Ddx58, Eif2ak2, Faslg, Ifih1, Il18, Il1a, Il21, Irf7, Ltb, Myd88, Nod2, Oas1, Oas1b, Oas2, Oas3, Ptx3, Tlr3, Tlr6, Tnf, Tnfsf10, Tnfsf12</i>
Systemic Lupus Erythematosus In B Cell Signaling Pathway	1.00×10^{-10}	<i>Cd72, Cd79a, Cd79b, Cntf, Faslg, Ifih1, Ifit2, Ifit3, Ighm, Il18, Il1a, Il21, Irf7, Irf9, Isg15, Isg20, Lilrb3, Ltb, Myc, Myd88, Pik3ap1, Rras, Shc1, Stat1, Stat2, Tlr3, Tnf, Tnfsf10, Tnfsf12, Traf1</i>
Interferon (IFN) Signaling	1.62×10^{-9}	<i>Ifi35, Ifit3, Ifitm3, Irf1, Irf9, Isg15, Oas1, Socs1, Stat1, Stat2, Tap1</i>
Th1 and Th2 Activation Pathway	8.32×10^{-9}	<i>Acvr1c, Aph1a, Cd274, Cd28, Cd3d, Cd3e, Cd3g, Cd8a, Cxcr6, Icam1, Icos, Il18, Il2rb, Irf1, Jak3, Klrd1, Socs1, Socs3, Stat1, Stat4, Stat6</i>
Acute Phase Response Signaling	1.70×10^{-8}	<i>A2m, C1r, C1s, C3, Hpx, Il18, Il1a, Il1rn, Itih3, Map2k3, Myd88, Rras, Serpina3, Serpinf1, Serpinf2, Serping1, Shc1, Socs1, Socs3, Tnf, Tnfrsf11b</i>

The module directly upstream of CFM long-term memory outcomes was m5, enriched for GO biological processes associated with viral immune response (**Figure 2.2**). This module consisted of 848 gene members, making it one of the largest modules constructed in this analysis. Enrichment of this module for GO biological processes and IPA canonical pathways highlighted the primary function of this module was pro-inflammatory immune signaling. Specifically, many of the enriched IPA canonical pathways referenced pathways involved in immune responses to virus, such as “role of pattern recognition receptors in recognition of bacteria and viruses”, “interferon signaling”, and “acute phase response signaling” (**Table 2.3**), confirming the primary function of this module as immune response. Upon further inspection of module members that also participated in these pathways, many are members of the interferon

response pathway (e.g. *Oas1*, *Oas1b*, *Oas2*, *Oas3*, *Eif2ak2*, and *Ifih1*) which is the primary pathway activated upon viral infection^{220,221}.

2.2.4 Genetic mapping of m7 module eigengene identifies *Fgf2* as a regulator of m7 and short-term memory outcomes

A major advantage of using genetic reference panels to model polygenetic disorders is the ability to perform genetic mapping of complex traits for such disorders, including measures of module expression, to identify chromosomal regions and genes within them that regulate these traits. To identify chromosomal regions regulating module expression we performed quantitative trait loci (QTL) mapping of module eigengenes for all cognitive relevant modules (**Figure 2.7**, depicted as green triangles). Specifically, we focused on identifying chromosomal regions regulating m5 and m7 expression, since these modules were identified through Bayesian modeling as being directly upstream of cognitive outcomes. While we did not identify any QTLs for the m5 eigengene, we identified a significant QTL for the m7 eigengene, which fell in a small region on chromosome 3 (36.5-53.3Mb), which means genetic variation within this region corresponds to variation in m7 expression (**Figure 2.8A**). Within this region of chromosome 3 we identified 45 protein coding genes, only one of which was a member of m7, Fibroblast growth Factor 2 (*Fgf2*), identifying this gene as a possible regulator of m7 expression and activity. Investigation of the variants in *Fgf2* between C57BL/6J and DBA2/J in the Sanger Mouse Genomes databased highlighted many single nucleotide polymorphisms (SNPs) between the two strains, the most notable being a possible splice donor and/or missense variant⁵¹. Additionally, our data show that hippocampal *Fgf2* expression is significantly higher in strains that carry the B allele compared to strains that carry the D allele at the marker SNP closest to the *Fgf2* gene body (**Figure 2.8B**). Finally, we found that *Fgf2* expression significantly negatively correlated with Y-

maze working memory outcomes in strains that carried the B allele at *Fgf2*, but carried no significant relationship with mice that carried the D allele at *Fgf2* (**Figure 2.8C**). Comparatively, *Fgf2* expression in all strains significantly negatively correlated with CFA short-term memory outcomes (**Figure 2.8D**). Together, this genetic mapping nominated a candidate by which m7 expression and possibly activity is controlled, and further experiments will determine the exact role by which *Fgf2* may control memory outcomes during aging and AD.

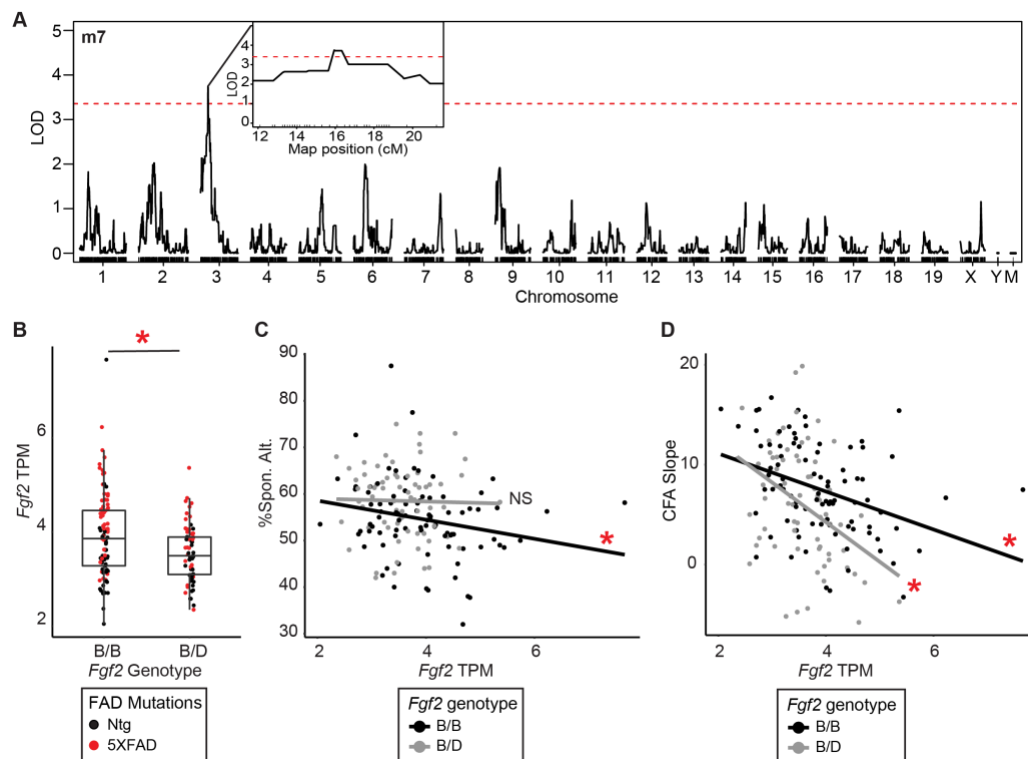


Figure 2.8: Mapping of the module upstream of working memory and short-term memory measures identifies *Fgf2* as a potential driver. A) Genetic mapping of m7 using the first principal component (PC1) as module eigengene. Age, sex, and fAD mutation status were used as additive covariates. B) Stratification of *Fgf2* expression based on genotype at the closest marker Single Nucleotide Polymorphism (SNP) to *Fgf2* genetic location (Student's t-test, $p < 0.05$). C) *Fgf2* expression is significantly correlated (Spearman, $p < 0.05$) with overall working memory performance only in mice with the B/B genotype at the marker SNP closest to *Fgf2* but not with B/D. D) *Fgf2* expression is significantly correlated (Spearman, $p < 0.05$) with overall short-term memory performance in mice with either the B/B or and B/D genotype at the marker SNP closest to *Fgf2*. Significant correlations denoted by red asterisks. NS = no significant correlation.

2.2.5 Molecular sub-networks for m5 implicate specific immune pathways and genes as modulators of long-term memory function

Since a large number of genes (848 genes) fell into m5, the module directly upstream of CFM long-term memory outcomes in the Bayesian network, we conducted a sub-clustering analyses to resolve smaller molecular networks that may exist within the larger module. Specifically, we performed SpeakEasy clustering analysis on gene expression measures from m5 members, and identified five distinct sub-clusters (SCs) within m5 (**Figure 2.9A**). GO enrichment of the genes within each SC revealed immunological pathways as the top enriched GO term, such as response to virus (SC1), lymphocyte differentiation (SC2), acute inflammation (SC3), Interleukin-8 (IL-8) secretion (SC4), and acrosome reaction (SC5). Spearman correlation of each SC to CFA slope short-term memory and CFM long-term memory outcomes highlighted significant negative module-trait correlations for each SC except for SC5, which was only correlated with long-term memory (**Figure 2.9B**). Furthermore, SC1: response to virus, SC3: acute inflammation and SC4: IL-8 secretion explained comparable variance in CFA slope short-term memory outcomes to m5 expression (**Figure 2.9C**), while SC3 and SC4 explained comparable variance in long-term memory outcomes to m5 expression (**Figure 2.9D**). Finally, while the m5 eigengene alone did not map to a QTL, we identified six QTLs for the SC5 eigengene (**Figure 2.9E**). The m5 gene members within these regions were enriched for immune functions, suggesting regions of the genome that contain genes associated with immune function could also be important for regulating how m5 controls long-term memory outcomes, since SC5 was also correlated with long-term memory. Furthermore, when we investigated how m5 SCs were genetically controlled in the AD-BXD population, we found that the SC5 eigengene mapped to a smaller region on chromosome 1 that overlapped with the QTL we identified in **Figure 2.9 (Figure 2.10C)**. This region of chromosome 1 was also

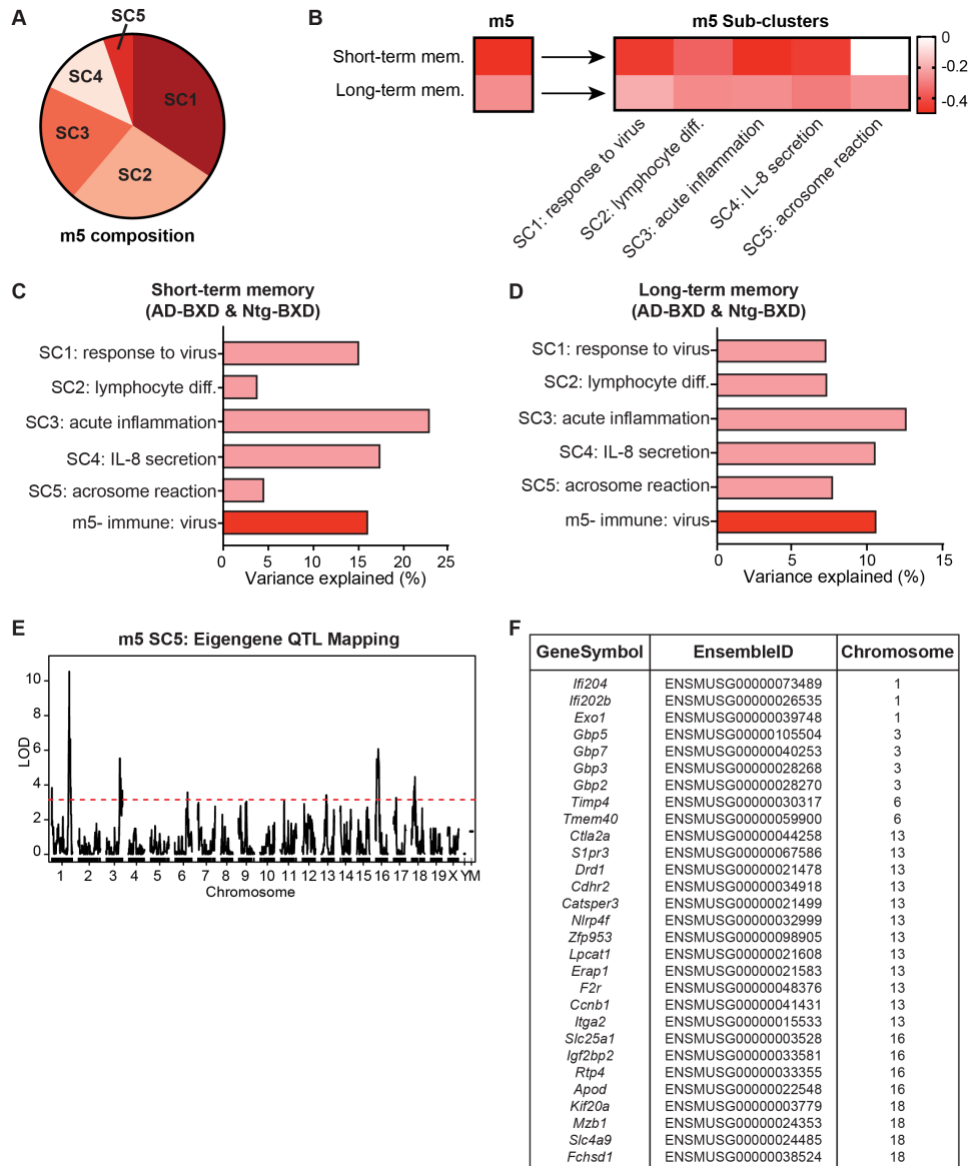


Figure 2.9: Sub-clustering of the m5 immune module identifies five sub-clusters with immune function and QTLs that map to immunologically-relevant genetic regions. A) Composition of m5 based on sub-cluster (SC) gene membership; for the 848 genes in m5 the sub-clusters broke out into 291 genes in SC1, 228 genes in SC2, 176 genes in SC3, 108 genes in SC4, and 45 genes in SC5. B) Spearman correlations (adj. $p < 0.05$) of m5 and SC1-SC5 to short-term and long-term memory measures. C-D) The proportion of variance in measures for short-term memory (C) and long-term memory (D) explained by m5 (dark red) and SC1-SC5 (light red) average expression. For B-D SCs are denoted by the most significantly enriched GO term. E) Genetic mapping of the first principal component (PC1) as module eigengene for SC5; Age, sex and fAD mutation status were used as additive covariates. F) Table denoting protein-coding genes symbols, ensemble IDs, and chromosomal location for each gene within the SC5 QTL peaks that were also m5 members.

enriched for m5 members associated with the interferon response pathway (*Ifi204*, *Ifi202b*), adaptive immune response (*Exo1*, *Fcrl6*) and glia signaling (*Kcnj10*) (**Figure 2.10D**). Together, this sub-clustering analysis further elucidates the overall function of m5 for viral and adaptive immune response, and nominates a region on chromosome 1 that could be important for controlling m5 expression. Future experiments will deduce the function of this genetic region, and how genes within may control m5 expression and possibly long-term memory outcomes.

2.2.6 The effect of module expression on AD-BXD long-term memory outcomes is partially modulated by amyloid accumulation

While the previous analyses were performed on the combined AD-BXD and Ntg-BXD population that contained individual strains that did not carry amyloid-driving mutations, many of the networks explaining large variance in cognition, such as inflammation (m4 and m5), related to pathways previously implicated in the etiology of AD. To evaluate how the presence of the 5XFAD transgene affected module expression, we employed three-way ANOVA tests on module expression, and found that 11 of the 16 cognitively relevant modules were significantly affected (adj. $p < 0.05$) by presence of the 5XFAD transgene (**Figure 2.3**), suggesting that the presence of amyloid-driving mutations accounts for the majority of the significant module-trait relationships. To test if module-trait relationships were primarily driven by the presence of fAD mutations, we recalculated the module-trait Spearman correlations of the 16 cognitively relevant modules for the Ntg-BXD and AD-BXD populations separately. While there were no significant module-trait correlations in the Ntg-BXD population, we found that 7 of the 16 original cognitively relevant modules continued to be correlated to cognitive outcomes in the AD-BXD population, including m5 (immune: virus) and m7 (lipid biosynthesis), which were the modules directly upstream of cognitive outcomes in the Bayesian model (**Figure**

2.11A). This supported the hypothesis that the presence of amyloid-driving mutations strengthens the module-trait relationships that explain a portion of the individual differences in cognitive outcomes.

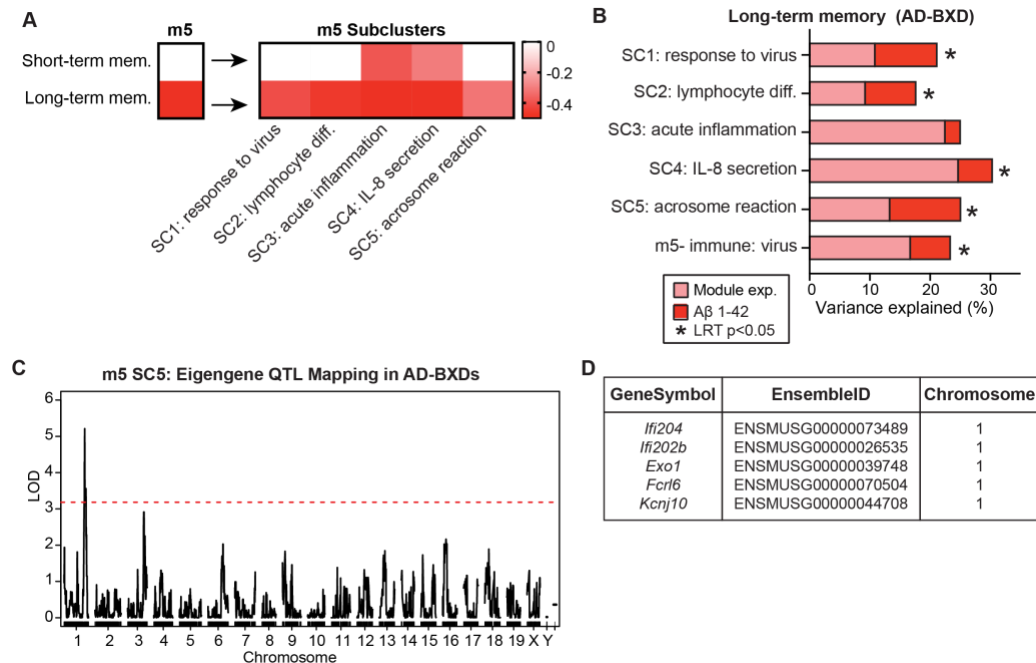


Figure 2.10: m5 sub-cluster AD-BXD relationships to long-term memory are strengthened by amount of amyloid, and map to a region on chromosome 1. A) Average standardized m5 sub-cluster (SC) expression for AD-BXD mice was correlated to short-term memory and long-term memory outcomes. SCs with no significant correlation are depicted in white. B) The proportion of variance in measures for long-term memory explained by SC expression and amount of amyloid in AD-BXD mice, using an additive Type 2 ANOVA model. Variance explained by SC expression for each module is in light red, while amount of variance explained by cortical A β 1-42 as part of the additive linear model is in dark red. Likelihood-ratio (LRT) tests were performed for each model to test if the addition of cortical A β 1-42 into the additive model generated a better fit of linear model. Significant p-values ($p < 0.05$) of LRT tests are denoted by black asterisks. All modules are denoted by the most significantly enriched GO term. C) Genetic mapping of the first principal component (PC1) eigengene for SC5 in the AD-BXD cohort; Age, sex and fAD mutation status were used as additive covariates. D) Table denoting protein-coding gene symbols, ensemble IDs, and chromosomal location for each gene within the SC5 AD-BXD QTL peaks that were also m5 members.

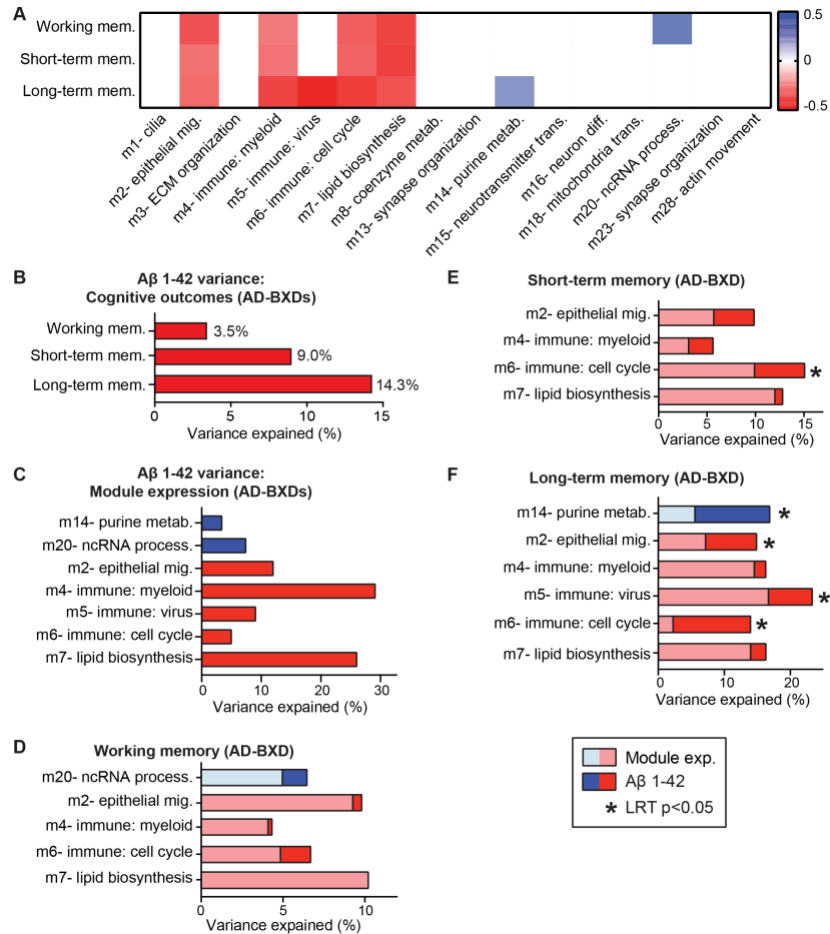


Figure 2.11: AD-BXD long-term memory module-trait relationships are impacted by the amount of amyloid accumulation. A) Average standardized module expression for 5XFAD mice was correlated to cognitive traits to identify modules that significantly modify cognitive function in the AD-BXD mouse population. Only the modules that were significantly correlated with cognitive function in the entire population were tested for significant relationships within the AD-BXD cohort. Modules with no significant correlation to corresponding cognitive outcome are depicted in white. B) The proportion of variance in cognitive outcomes explained by amount of cortical amyloid (quantified by Aβ1-42-specific sandwich colorimetric ELISA) in AD-BXD mice. C) The proportion of variance in module expression explained by amount of cortical Aβ1-42. D-F) The proportion of variance in measures for working memory (D), short-term memory (E), and long-term memory (F) explained by module expression and amount of cortical Aβ1-42 in AD-BXD mice, using an additive Type 2 ANOVA model. Variance explained by individual modules significantly positively (red) and negatively (blue) correlated to corresponding cognitive trait are in lighter colors, while amount of variance explained by amyloid as part of the additive linear model is in darker colors. Likelihood-ratio (LRT) tests were performed for each model to test if the addition of cortical Aβ1-42 into the additive model generated a better fit of linear model. Significant p-values (p<0.05) of LRT tests are denoted by black asterisks, indicating the addition of cortical Aβ1-42 to the model provides a better linear fit than module expression alone. All modules are annotated by the most significantly enriched GO term (NS GO = no significant GO enrichment).

To determine the degree to which the 7 significant module-trait relationships within the AD-BXD population were dependent on the amount of brain amyloid (**Figure 2.11A**), we quantified the amount of variance that cortical amyloid load explained for each cognitive measure within the AD-BXD population (A β 1-42 ELISA assay previously reported in Neuner⁴⁸). Cortical A β 1-42 (ng/mg) explained 3.5% of working memory (not statistically significant), 9.0% of short-term memory ($p < 0.05$), and 14.3% of long-term memory measures ($p < 0.01$) (**Figure 2.11B**). For several modules A β 1-42 could explain a large amount of the variance in module expression, such as 29.4% of the variance in m4 expression, and 26.3% of the variance in m7 expression ($p < 0.01$) (**Figure 2.11C**). This suggested that A β 1-42 likely influences the expression of modules within AD-BXDs.

To test the degree to which cortical A β 1-42 modulated the effect of each module (as measured by average expression) had on each cognitive domain, we generated linear models for each significant module-trait relationship, with the addition of cortical A β 1-42 ELISA measures as a second independent variable within the model. We first employed Type 3 ANOVA statistical tests to obtain significant module-A β 1-42 interactive effects on cognitive outcomes that identified only one module-A β 1-42 relationship (m4) had a significant interactive effect on Y-maze %SA (**Table 2.4**). Since only a single interactive effect was obtained, we tested the additive effect of module expression and cortical A β 1-42 accumulation using Type 2 ANOVA statistical tests (**Table 2.4**), as well as likelihood-ratio (LRT) tests to determine if the addition of amyloid into the model significantly improved the linear fit that explained cognitive outcomes using module expression²²². Within AD-BXD individuals, module expression for each significantly correlated module explained up to 10% of the variance in Y-maze working memory %SA outcomes, and the models for this cognitive outcome were not significantly improved by the addition of A β 1-42 accumulation (**Figure 2.11**). This was similar for CFA short-term memory outcomes, except the relationship of m6 to CFA slope was significantly improved by the addition of

Table 2.4: ANOVA results from AD-BXD mice using interactive and additive models for the effects of module expression and amount of amyloid on cognitive outcomes.

Independent variable (module or amyloid)	Dependent variable (trait)	Type 3 ANOVA p-value	Type 3 ANOVA variance	Type 2 ANOVA p-value	Type 2 ANOVA variance
m2	Y-maze	0.02796	6.9963962	0.01078	9.2616462
Amyloid		0.02796	1.0346444	0.52689	0.5446697
m2*Amyloid		0.53233	0.5458877	--	--
m4	Y-maze	0.0053	9.740127	0.09526	4.0891733
Amyloid		0.02349	6.299756	0.68826	0.2318769
m4*Amyloid		0.01997	6.660873	--	--
m6	Y-maze	0.02928	6.444308	0.06654	4.84505
Amyloid		0.0583	4.8193	0.25419	1.842201
m6*Amyloid		0.12887	3.069617	--	--
m7	Y-maze	0.007338	10.10619	0.007453	10.20718031
Amyloid		0.353488	1.152785	0.977726	0.00105253
m7*Amyloid		0.274227	1.60491	--	--
m20	Y-maze	0.4648	0.7980078	0.06369	4.963853
Amyloid		0.5625	0.5003325	0.30477	1.49321
m20*Amyloid		0.3603	1.252871	--	--
m2	CFA-Slope	0.02625	6.703622	0.04205	5.765807
Amyloid		0.03606	5.93877	0.07763	4.310485
m2*Amyloid		0.24794	1.762245	--	--
m4	CFA-Slope	0.1313	3.3326177	0.1389	3.153778
Amyloid		0.25	1.9228184	0.173	2.666946
m4*Amyloid		0.5427	0.5343766	--	--
m6	CFA-Slope	0.01583	7.747088	0.006564	9.953652
Amyloid		0.02505	6.640194	0.043868	5.334866
m6*Amyloid		0.18618	2.253916	--	--
m7	CFA-Slope	0.004101	11.360047	0.003311	12.0710144
Amyloid		0.183817	2.317112	0.440296	0.7838669
m7*Amyloid		0.275075	1.555718	--	--
m2	CFM	0.02701	6.5214804	0.02039	7.169561
Amyloid		0.01269	8.3696007	0.01632	7.712189
m2*Amyloid		0.38564	0.9723399	--	--
m4	CFM	0.01249	9.050469	0.0011	14.542526
Amyloid		0.59716	0.3869789	0.2445	1.722808
m4*Amyloid		0.99143	0.0001596	--	--
m5	CFM	0.00433	10.602513	0.0002954	16.69156
Amyloid		0.01379	7.774235	0.0188263	6.632697
m5*Amyloid		0.27209	1.489265	--	--
m6	CFM	0.572	0.46531146	0.190902	2.241522
Amyloid		0.0884	4.31511548	0.003545	11.732871
m6*Amyloid		0.9727	0.00170564	--	--
m7	CFM	0.01123	9.2273761	0.001347	13.986886
Amyloid		0.3559	1.172067	0.178273	2.311905
m7*Amyloid		0.77991	0.1067465	--	--
m14	CFM	0.20409	2.2290731	0.038213	5.547295
Amyloid		0.01574	8.3260918	0.003583	11.311853
m14*Amyloid		0.88972	0.0262483	--	--

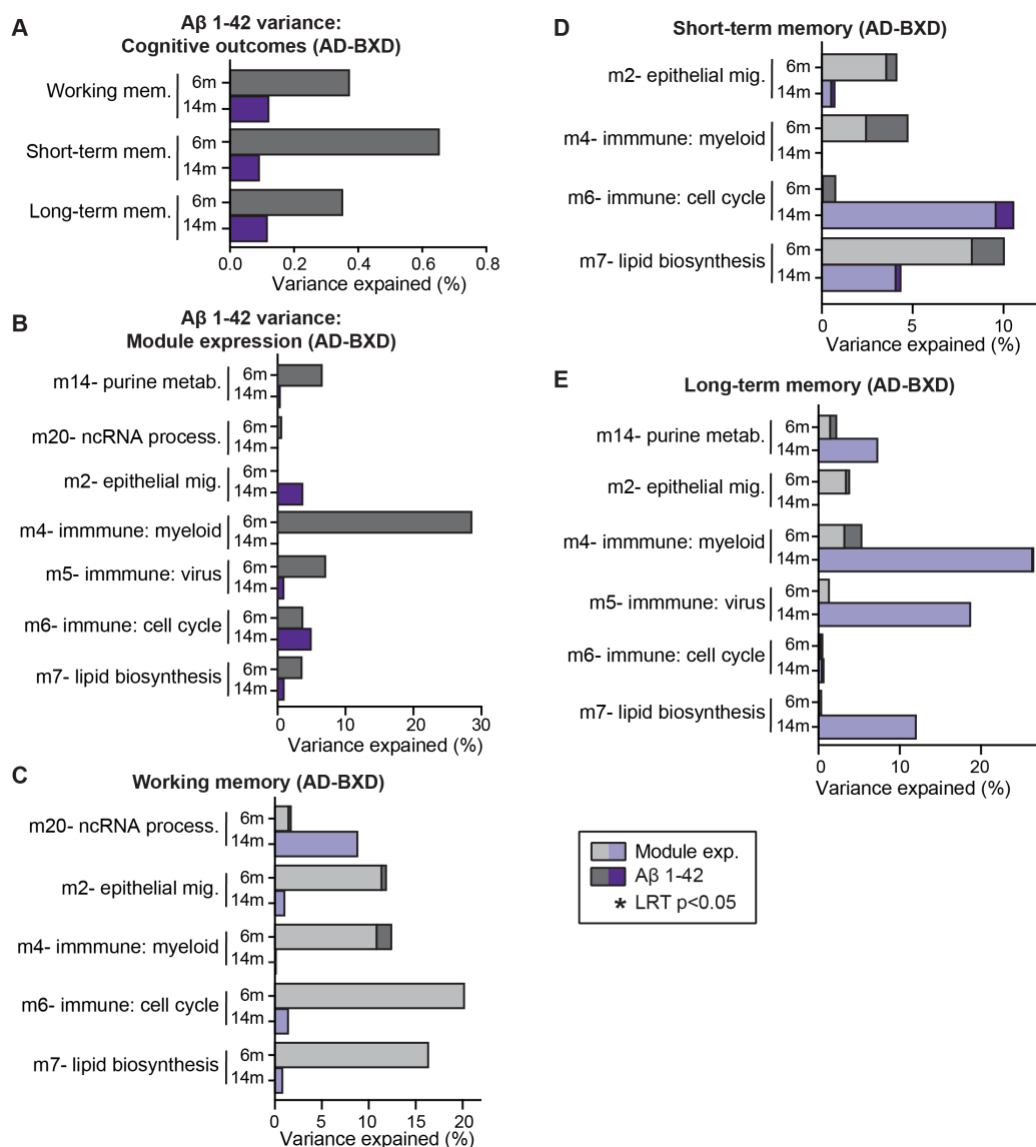


Figure 2.12: Variance explained in cognitive outcomes from AD-BXD mice separated by age. Modules significantly correlated to cognitive outcomes in the AD-BXD population (**Figure 5A**) were separated by age to test for amount of variance explained by module expression and cortical $A\beta$ 1-42 within young and aged mice. Bars in grey are results from 6m AD-BXDs, and bars in purple are results from 14m AD-BXDs. A) The proportion of variance in cognitive outcomes explained by cortical amyloid (quantified by $A\beta$ 1-42-specific sandwich colorimetric ELISA) in AD-BXD mice. B) The proportion of variance in module expression explained by cortical $A\beta$ 1-42. C-E) The proportion of variance in measures for working memory (C), short-term memory (D), and long-term memory (E) explained by module expression and cortical $A\beta$ 1-42 in AD-BXD mice, using an additive Type 2 ANOVA model. Variance explained by module expression are in lighter colors, while amount of variance explained by cortical $A\beta$ 1-42 as part of the additive linear model is depicted in darker colors. All modules are denoted by the most significantly enriched GO term (NS GO = no significant GO enrichment). LRT tests were performed but no model was significantly improved by addition of cortical $A\beta$ 1-42 to the linear fit.

A β 1-42 accumulation into the linear model (**Figure 2.11E**). One thing to note is that the effect of m7, the module upstream of working memory and short-term memory in the Bayesian model (**Figure 2.7**), on cognitive outcomes was not improved by the addition of A β 1-42 accumulation to the linear model, suggesting a nominal role for how amyloid influences these module-trait relationships. Finally, for CFM long-term memory outcomes, the effect of all except for two modules (m4, m7) was significantly improved by the addition of A β 1-42 into the linear model, suggesting that amyloid may play a more direct role on influencing the module-trait relationships for modules associated with long-term memory (**Figure 2.11F**). Additionally, all except one of the m5 SCs were significantly improved by the addition of A β 1-42 into the linear model (**Figure 2.10A-B**), suggesting that the specific relationship between m5 (and subsequent SCs) and long-term memory outcomes are influenced by amyloid accumulation. To gain a further understanding of the age-related factors that could drive these relationships, we re-created linear models within 6m AD-BXD and 14m AD-BXD (**Figure 2.12**). Interestingly, while the majority of the variance for working memory and short-term memory outcomes were observed within the 6m AD-BXD cohort, module expression explained more variance in 14m AD-BXD for long-term memory outcomes (**Figure 2.12**). This indicates that early-in-life perturbation of m7 at 6m or earlier may have a greater effect on altering working and short-term memory cognitive domains, while perturbation of m5 at a later age (14m) may prove more successful to improve the long-term memory cognitive domain. Together, these findings suggest a specific role of the interaction of cortical A β 1-42 accumulation and module expression on influencing long-term memory outcomes, while a more indirect or nominal role of A β 1-42 accumulation on influencing how modules affect working memory and short-term memory outcomes.

2.2.7 Cognitively relevant mouse modules replicate in aged human molecular brain networks

A criticism of using mouse models for AD research is that many previous findings in mouse models have failed to translate to human patients. Therefore, we tested the replication of all mouse co-expression modules in human brain data, including those predicted to be upstream of variation in cognitive outcomes across our mouse population. Using a consensus genetic clustering analysis of dorsolateral pre-frontal cortex (DL-PFC) from aged individuals (AD and non-AD)⁷⁸, we performed statistical hypergeometric tests to see how well our mouse modules overlapped with those found in humans. We found that the majority (15/16) of the cognitively relevant mouse modules were significantly (adj. $p < 0.05$) conserved (showed significant overlap) in human DL-PFC modules (**Figure 2.13**). The only non-conserved module was m6 (immune/cell cycle), which was surprising since indicators of inflammation are consistently observed in AD tissue. However, other immune-related modules were conserved, while the lack of conservation for this module may be due to its mixture of innate immune response genes and cell cycle genes. We also tested the conservation of molecular networks generated by a meta-analysis of multiple brain regions from human aged and AD tissue⁷⁷, and found that all 16 significantly correlated mouse modules in at least one brain gene network (**Figure 2.14**). Specifically, all of the significantly correlated mouse modules were conserved in the DL-PFC molecular networks from the Accelerating Medicines Partnership- Alzheimer's Disease (AMP-AD) consortium meta-analysis molecular networks, strengthening our findings from the conservation with the Mostafavi DL-PFC networks. Notably, the two prioritized modules from our Bayesian analysis (m5 and m7) were both significantly conserved with several human modules associated with cognitive function across the population (**Figure 2.13 & Figure 2.14**). These results highlight that both the major molecular networks and ontology pathways observed in a

genetically diverse mouse population are also replicated in human cohorts, making our current findings prioritizing m5 and m7 relevant to human AD and aging populations.

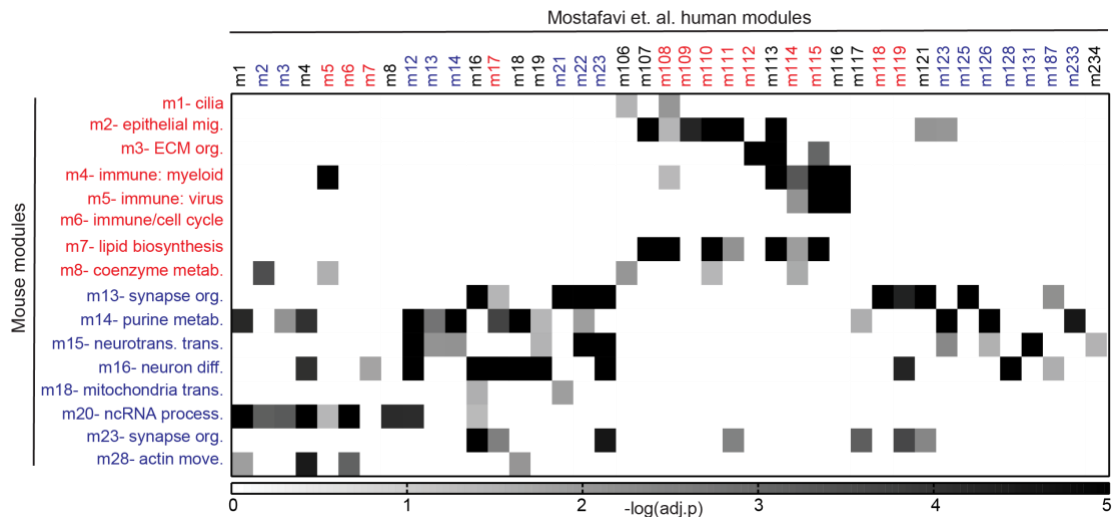


Figure 2.13: Mouse modules significantly associated with cognitive outcomes have high degree of conservation to human AD modules. Mouse modules significantly correlated to cognitive measures (left, y-axis) were examined for gene conservation in previously established (Mostafavi *et al.*, 2018) aged/AD human dorsolateral pre-frontal cortex (DL-PFC) modules (top, x-axis). Modules are colored based on positive (red) or negative (blue) correlation to corresponding mouse (working/short-term/long-term memory) or human (slope of decline) cognitive measure. Heatmap depicts degree (measured by $-\log[\text{adj. p-value}]$) of overlap (hypergeometric test) between human and mouse modules. Only significant (adj. p-value < 0.05) overlaps are depicted, while non-significant overlaps are white. Human modules were excluded if they did not overlap with at least one mouse module tested above. Mouse modules are denoted by the most significantly enriched GO term.

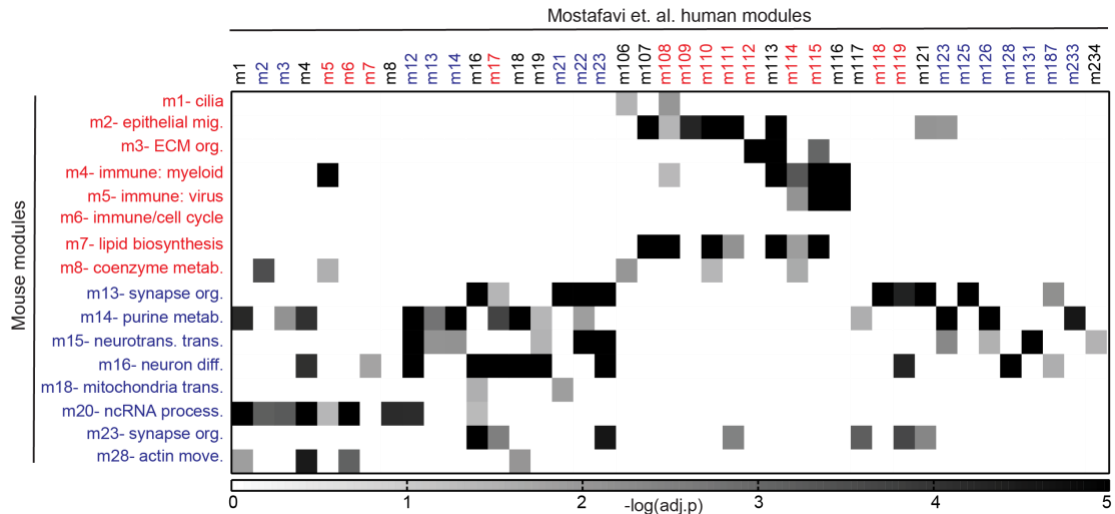


Figure 2.14: Mouse modules significantly associated with cognitive outcomes have high degree of conservation to human AD modules. Mouse modules significantly correlated to cognitive measures (left, y-axis) were examined for gene conservation in previously established (Mostafavi *et al.*, 2018) aged/AD human dorsolateral pre-frontal cortex (DL-PFC) modules (top, x-axis). Modules are colored based on positive (red) or negative (blue) correlation to corresponding mouse (working/short-term/long-term memory) or human (slope of decline) cognitive measure. Heatmap depicts degree (measured by $-\log[\text{adj. p-value}]$) of overlap (hypergeometric test) between human and mouse modules. Only significant (adj. p-value < 0.05) overlaps are depicted, while non-significant overlaps are white. Human modules were excluded if they did not overlap with at least one mouse module tested above. Mouse modules are denoted by the most significantly enriched GO term.

2.3 Discussion

2.3.1 Systems genetics identifies causal module-trait relationships in a population of AD mice that explain individual differences in cognitive outcomes

The search for the underlying genetic and molecular causes of AD cognitive decline has been the primary goal for identifying and developing therapeutics for several decades²²³. Identification of such genetic and molecular mechanisms that influence AD symptoms using preclinical mouse models has been criticized because of the poor translatability of findings in mouse models to effectively treating human AD^{224,225}. Modeling the disease in more genetically diverse mice may be one avenue to improve the construct validity of preclinical models^{48,226}. Using this model, we were able to

leverage transcriptomic and phenotypic information from pre- and post-symptomatic mice with and without AD mutations to identify the molecular networks that are predicted to directly influence variation in cognitive outcomes across a population. We found many sets of co-expressed genes, each representing the activity of one or more molecular systems, that were significantly correlated with population-wide differences in cognition, and explained up to 30% of the variance in cognitive outcomes. This is important because while high-impact variants such as those in *APOE* can explain a considerable proportion of AD risk, many of the more common AD variants have much smaller effect-sizes making new gene targets less impactful for translation to larger populations^{78,227,228}. In contrast, the upstream molecular networks identified here explain larger variances and may have a much larger effect on improving cognitive outcomes on the population levels. The networks consistently explaining the most variance were enriched for viral and myeloid immune pathway genes (m4, m5, m6), as well as lipid and coenzyme metabolism pathway genes (m7, m8). Additionally, genes within these biologically significant modules were found to be primarily expressed in non-neuronal cell-types such as astrocytes and microglia, suggesting that regulation of the specific pathways [lipid biosynthesis, IL-1 signaling, interferon (IFN) signaling] within these cell types contributes most significantly to divergent cognitive outcomes across resilient and susceptible individuals²¹³. These observations were further substantiated by our Bayesian modeling of module-trait relationships in that the only modules directly upstream of cognitive outcomes were enriched for non-neuronal genes: the astrocyte lipid biosynthesis module (m7) for working and short-term memory, and the microglia viral immune module (m5) for long-term memory. This model proposed two cell-type enriched molecular networks to prioritize for further investigation, since our model predicted that population-wide differences in these networks have greatest impact on regulating cognitive outcomes. A critical aspect of cell-type enrichment analyses

performed on bulk RNAseq data used here is while they suggest biological pathways that could be altered in enriched cell-types, many “cell-type specific” genes are non-autonomously expressed across the CNS and will require further investigation to validate the pathway changes discussed here are specific to a particular cell population.

2.3.2 Targeting astrocyte lipid biosynthesis and IL-1 signaling may promote resilience to working memory and short-term memory decline

The gene network that our model found to be directly upstream of working and short-term memory was m7, which was enriched for “lipid biosynthesis” GO biological pathways. Notably, m7 was exclusively enriched for genes found in astrocytes, specifically, implicating astrocytic lipid biosynthesis as critical for determining cognitive outcomes. Further IPA analysis also highlighted a notable m7 gene, *Rhoa*, that emerged in three of the five top enriched canonical pathways (**Table 2.2**). This gene is of particular interest because therapeutically targeting RhoA pathways has been found to be promising for improving AD and mixed dementia symptoms, proposing a method by which targeting m7 could influence AD cognitive outcomes^{229–231}. While these previous studies have investigated RhoA pathway inhibition in the context of amyloid and tau pathology in AD and mixed dementia, these findings could suggest that inhibition of m7 via the RhoA pathway could also improve cognitive outcomes. Additionally, our IPA analysis identified other pathways of interest, such as “IL-1 signaling”, and “cholesterol biosynthesis.” IL-1 signaling in astrocytes plays an important role in promoting inflammation. IL-1 signaling deficiency has also been demonstrated to have detrimental effects on hippocampal synaptic plasticity^{232,233}. As the IL-1 signaling pathway is also an indicator of inflammation, the overall representation of this module could be for reactive (or A1) astrocytes, which have been widely described as being induced by inflammatory insult^{234–236}. More specifically, reactive astrocytes are thought to be induced by pro-

inflammatory cytokines TNF α , IL-1, and C1Q, which are highly expressed in microglia in the CNS^{75,213}. Two of the three transcripts for these cytokines appear in m5 (*Tnf*, *Il1a*), suggesting an interplay between these two modules and representative cell-types that directly influence cognitive resilience. Finally, an indicator of inflammation that has been characterized in astrocytes and microglia is the formation of lipid droplets within these cells, which synthesizes with our other finding of additional enrichment of lipid metabolism pathway genes in this module^{237,238}.

In addition to the interesting pro-inflammatory pathways, the “cholesterol biosynthesis” pathway present in m7 suggests an additional mechanism by which astrocytes may dictate cognitive outcomes. Cholesterol biosynthesis has been previously implicated in AD pathogenesis due to a variety of mechanisms. For example, cholesterol influences the relationship between amyloid precursor protein (APP) and in the production of A β , but the mechanism by which this occurs is still not understood well²³⁹. In addition, cholesterol biosynthesis is of particular importance to mature neurons since astrocytes are the primary cholesterol source for mature neurons in the CNS^{240,241}. A negative association between lipid biosynthesis and cognitive outcomes could indicate a stall or dysfunction in this process by either depriving neurons of necessary lipids or over-production within astrocytes. Finally, *APOE*- the most significant risk factor for sporadic AD- is also intimately involved in cholesterol metabolism. Risk variants in *APOE* have been hypothesized to disrupt the functional ability of APOE to bind cholesterol and therefore contribute to AD pathogenesis^{242,243}. As the BXD panel contains naturally occurring variation in the *Apoe* receptor binding domain (possibly altering protein function across strains carrying the B versus D allele of this gene), we may have identified the cell-type and pathway by which *Apoe* variation influences cognitive resilience^{23,48,244}. Further studies will be conducted to 1) test if lipid biosynthesis is higher in astrocytes from mice that perform worse on short-term and/or working memory

cognitive tasks using pulse-chase experiments^{245,246}, and 2) observe if the IL-1 inflammatory signature from this module synthesizes with lipids by evaluating lipid droplet formation within astrocytes from AD-BXD individuals using BODIPY staining²⁴⁷. These experiments will further our understanding of the contribution of specific pathways we believe to be involved in astrocytes that help dictate AD cognitive outcomes.

2.3.3 *Fgf2* as a possible regulator of working and short-term memory outcomes

In this study we leveraged the power of the known genetic composition of our AD-BXD panel to identify a chromosomal region, and a protein-coding gene within, that regulates the expression of m7, the module directly upstream of Y-maze working memory and CFA short-term memory outcomes. The nominated gene, *Fgf2*, showed a strong negative overall relationship of expression with short-term memory outcomes. The FGF2 protein has a variety of known functions in the CNS, including promoting angiogenesis, neurogenesis, and improving symptoms of cognitive aging²⁴⁸. FGF2 is primarily expressed in astrocytes in the adult brain, but can also be secreted by neurons when damaged to produce a distress signal to recruit microglia and astrocytes to the site of damage^{249,250}. Functionally, FGF2 is known to regulate the proliferative response to oligodendrocyte precursor cells (OPCs) during remyelination of axons after acute demyelination injury, suggesting another role of FGF2 signaling in regulating glia-neuron homeostasis^{251–253}. Experimentally-induced spinal cord and CNS injury has shown to promote reactive astrogliosis, and these reactive astrocytes have been previously shown to up-regulate FGF2, suggesting an additional involvement of FGF2 in the proliferation and reactivity of astrocytes to sites of injury and recovery²⁵⁴. Increased FGF2 (or basic FGF, bFGF) has been observed in reactive astrocytes and the neurons surrounding amyloid plaques, as well as its localization to neurofibrillary tangles and cerebrovascular deposits in AD patient brain tissue^{255–257}. However, delivery of FGF2 into the

hippocampus of APP mice using AAV2/1 viral construct caused mice to show enhanced long-term potentiation, reduced A β production, and enhanced neurogenesis, suggesting FGF2 can act to halt AD pathophysiology²⁵⁸. Together, while our data suggest that more *Fgf2* is detrimental to cognitive performance, other data suggest FGF2 activity is important to glial and neuronal response to injury or pathological environment. However, characterization of bFGF with AD pathology suggests it could be part of the reactive astrocyte response to disease activity. Further work will have to be conducted to determine the cell-type for which *Fgf2* is promoting a negative correlation with cognitive outcomes, the cellular pathways by which it is acting, and how it could be detrimental to AD cognitive outcomes.

2.3.4 Targeting viral and adaptive immune pathways to promote long-term memory resilience

The robust associations of microglia and microglial processes with improving cognitive outcomes in mice is consistent with genetic^{18,259} and cellular data^{139,142}, indicating their relevance to AD disease activity and cognition^{115,260}. Surprisingly, when we interrogated our molecular networks in search of known AD risk genes enriched in immune processes, and found that the majority of the sporadic AD immune-related risk genes were actually present in m4, our immune: myeloid module, and not m5, our immune: virus module. It was interesting that several high impact sporadic AD genes (*BIN1*, *TREM2*, *APOE*) were present in a single module (we allowed discrete assignment of each gene to one module in this study), and also that this module did not explain as much variance in cognitive outcomes as the viral immune pathway, with m4 explaining 6.6% and m5 explaining 10.5% of the variance in long-term memory (**Figure 2.6**). It could be possible that these m4 LOAD genes and immune: myeloid pathways in

m4 have a more indirect effect on AD cognitive resilience as a molecular system, compared to the viral immune response pathway.

Given recent evidence that viral immune pathways, including the type-1 interferon (IFN) pathway, are up-regulated in human AD patients, the viral immune network identified in m5 is an interesting pathway associated with the neuroinflammatory response observed in AD²⁶¹. Previous studies using mouse models of AD have demonstrated that knock-out of the type-1 IFN receptor (IFNAR), an important receptor for the viral immune response, causes reduced amyloid monomer levels, and partial rescue of performance on the Morris water maze task at 9 months of age²⁶². Recently, conflicting evidence has emerged about the implications of viral infections, such as the presence of herpesviruses (HSVs, HHVs) in post-mortem brain tissue, with AD cognitive deficits^{263,264}. While these infections would not be present in the mice used in this study, since they are raised and aged in a controlled low-pathogen environment, it is interesting that we still observe activation of antiviral pathways. Additionally, we found very little overlap in m5 genes with genes that drive network activity of cells that are infected with herpesviruses such as HSV-1 or HHV6A found in AD patient brains^{264,265}. This suggests that the networks activated by these viruses in human patients are independent of the viral immune pathways enriched in m5, and that the m5 network represents an immune response to non-viral pathological burdens present in the brain, such as A β . This is supportive of our finding that the effect of m5 on long-term memory outcomes was improved by the addition of cortical A β accumulation in the linear model. However, since the mice used in this study have not been exposed to a systemic viral challenge, it could be possible that activating antiviral pathways systemically, by either HSV infection or poly I:C challenge, could activate other molecular networks that explain more variance in long-term memory outcomes compared to those activated in mice raised in a sterile environment. More work needs to be done in this field to decipher a specific interplay

between environmental factors (such as viral exposomes and diet), amyloid and the relevance of their interaction in AD pathogenesis²⁶⁶.

Along with viral immune pathways being represented in m5, our sub-clustering analysis highlighted more distinct immune pathways that continued to be correlated with long-term memory measures (**Figures 2.11 & 2.12**). The enriched pathways revealed by these sub-clusters further supported the importance of viral immune response pathways (represented in SC1), as well as indicators of an adaptive immune response (lymphocyte differentiation represented in SC2), and microglial migration (represented by IL-8 signaling in SC4). These pathways were interesting because while the importance of viral immune pathways remained, we also identified other pathways such as the IL-8 signaling pathway that we would not have identified as the top enriched pathway in the large m5 module. The IL-8 signaling pathway is interesting in the context of AD because IL-8 (with the official gene symbol of *Cxcl8*) is known to induce microglial migration, and has been previously found to increase in microglia exposed to amyloid^{267,268}. Additionally, IL-8 has been proposed as a biomarker for AD cognitive impairment and cerebrovascular dysfunction when it was found to be up-regulated in blood serum of AD and cognitively impaired individuals with cerebrovascular disease compared to non-cognitively impaired counterparts²⁶⁹. Therefore, enrichment of this pathway could be a possible indicator of population-wide differences in microglial amyloid migratory response, and differences in cerebrovascular dysfunction across the AD population. We also may have identified a measurable marker of resilience and susceptibility to cognitive impairment by identification of this m5 sub-cluster which we will test in future studies for validation. Finally, sub-clustering of m5 also allowed for genetic mapping of the SC5 eigengene to a region on chromosome 1 within the AD-BXD population (**Figure 2.9E-F & Figure 2.10C-D**). This region was interesting because several genes within this locus were also m5 members, and associated with IFN signaling (*Ifi204*, *Ifi202b*),

adaptive immune response (*Exo1*, *Fcrl6*), and a glia-specific potassium channel, *Kcnj10* (also known as Kir4.1). The region identified on chromosome 1 is incredibly interesting because of its enrichment for these glia and immune-related genes that were also m5 members, as well as inspection of these genes with the Sanger Mouse Genomes database reveals several coding variants between C57BL/6J and DBA/2J mice⁵¹. Additionally, SC5 was only significantly correlated with long-term memory outcomes, suggesting a specific role of this SC on integrating overall m5 activity with individual differences in long-term memory outcomes. Future work will elucidate how this SC5 molecular network, the chromosome 1 QTL, and the genes within it possibly control overall m5 activity and long-term memory outcomes.

Overall, our data and suggest that the specific pathways that will have the greatest impact on improving cognitive outcomes are those involved in the microglial viral immune and migratory response. It is still unclear if this module is representative of the local brain immune response by activation of microglia, or if this pathway is also comprised of infiltrating immune cells that can also contribute to the neuroinflammatory response in later stages of AD, which was proposed by the enrichment of lymphocyte differentiation genes in m5 SC2²⁷⁰. Additionally, the distinction between peripheral and local neuro-immune responses in the brain are difficult since microglia and peripheral macrophages express many of the same marker genes and proteins. Although our Bayesian model has found that the neuroinflammatory response is the major contributor to long-term memory resilience in our aging model, further experiments will be done to distinguish how much of this resilience network is dictated by peripheral or local innate immune responses. This will include investigating the role of the systemic immune system, and how increased infiltrating systemic immune cells (CD4+ T cells, B cells, peripheral macrophages) may play a negative role in cognitive resilience by evaluating brain gene and protein expression of these peripheral markers in this mouse population

and comparing to TMEM119+ microglia. Specifically, we hypothesize from these data that inhibition of the type-1 IFN signaling pathway in more susceptible mice will promote cognitive resilience by delaying the neuroinflammatory response that causes cognitive decline in AD. Additionally, we will further investigate the role of IL-8 as a possible biomarker of neuroinflammatory activity in susceptible AD individuals. Finally, we have proposed several gene candidates from QTL mapping of the m5 SC5 eigengene that we will examine as possible targets to promote resilience to AD long-term memory deficits.

2.3.5 Elucidating the contribution of A β accumulation to the impact of molecular networks on cognitive outcomes

As we have stated previously, AD is classified by progressive and severe memory decline, as well as the pathological presence of beta-amyloid (A β) plaques and neurofibrillary tau tangles¹⁹⁵. It is still widely debated if amyloid is directly involved in determining AD cognitive trajectories, or if it is more of associated pathology with limited involvement in cognitive decline²⁷¹. We previously found in our AD-BXD population that while cortical A β 1-42 levels were variable across genetic backgrounds (especially at 14m), this variability did not directly correlate with cognitive function⁴⁸. Here, we found that many of our identified module-trait relationships were strengthened when we isolated and tested these relationships in the AD-BXD population (**Figure 2.11A**), indicating the presence of fAD mutations is necessary for these molecular networks to influence cognitive outcomes. Using additional linear modeling, we tested the effects of cortical A β 1-42 accumulation on module expression, and if it could improve the influence of module expression on cognitive outcomes. Our results indicated a domain-specific effect that working memory and short-term memory were primarily influenced by module expression, and largely independent of individual differences in amyloid burden (**Figure 2.11D-E**). However, for long-term memory cortical A β 1-42 accumulation improved the effect of module expression on memory outcomes, indicating an indirect role of amyloid

on cognition through influencing module expression (**Figure 2.11F & Figure 2.10B**).

Therefore, we hypothesize that the role of A β on AD cognitive trajectories could be more complex than initially anticipated, and dependent on the cognitive domain and molecular pathway. It is possible that more module variance could be explained if we had measured and compared hippocampal A β 1-42 accumulation to gene expression changes instead of cortical A β 1-42, and are working to test this difference in future studies using A β 1-42 staining of fixed hemibrains from the same mice investigated here. Further studies will also test this model to see how perturbation of certain molecular networks influences cognitive outcomes, and if these networks will alter amyloid accumulation or if secondary amyloid targeting is also needed to have maximum effect, especially for long-term memory outcomes.

2.3.6 Advantages of diverse mice for defining the genetic origins of resilience

In this study, we were able to leverage previously generated transcriptome and phenotype information from a population of genetically diverse mice to identify the molecular networks that directly influence population-wide differences in cognitive outcomes. An advantage of using a genetic reference panel such as the BXDs is that we could leverage the well-defined genetics of this population to perform genetic mapping that anchored many of our module-trait relationships.

In addition to identifying immune and lipid metabolism pathways, as well as the *Fgf2* gene, as being possible direct regulators of AD cognitive outcomes, this study also identified neuronal and synaptic modules that were correlated to short-term memory outcomes (see **Figure 2.6**). We wanted to highlight these neuronal pathways in particular because recent studies using AD patient brain tissue have found that changes in dendritic spine morphology can indicate resilience to AD cognitive decline^{272,273}.

Further studies will test the importance of neuronal pathways in providing AD resilience, especially in the context of susceptible compared to resilient microglia or astrocyte environments, since targeting neuronal pathways have also been suggested to be desirable for therapeutic intervention in AD^{229,230}. Future work overlaying gene expression from mice that receive such interventions will also highlight how these molecular networks interact with each other when a specific module or target is perturbed. Finally, analysis of gene expression from this model system and comparison to human brain networks has nominated conserved molecular systems that are relevant to diverse AD cognitive outcomes. The identification of these relevant molecular systems will lead to a better understanding of preclinical models of human AD, which will allow for testing of novel resilience-based therapeutic strategies to improve AD.

2.4 Methods

2.4.1 Bioethics

All mouse experiments occurred at University of Tennessee Health Science Center and were carried out in accordance with the principals of the Basel Declaration and standards of the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), as well as the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center.

2.4.2 Animals

All data used in this study are from a mouse model of human AD, the AD-BXD panel, which were previously described⁴⁸. Briefly, female B6 mice hemizygous for the 5XFAD

transgene (B6.Cg-Tg(APP^{SweF1Lon}PSEN1*^{M146L}*^{L286V})6799Vas/Mmjax, Stock No. #24848-JAX) were mated to males from the BXD genetic reference panel^{274,275}. One generation of breeding B6-5XFAD dams with BXD sires results in a set of 26 isogenic F1 AD-BXD strains that either harbor the 5XFAD transgene or are non-transgenic (Ntg)-BXD littermate controls. Male and female AD-BXD mice were group housed as a mix of 5XFAD and non-transgenic same-sex littermates and maintained on a 12 hour light/dark cycle.

2.4.3 Y-maze

Behavioral testing to assess mice for working memory function was performed as previously described⁴⁸. In brief, mice were habituated to transport and to the testing room for three days prior to testing. The y-maze test of spontaneous alternation was performed for 8 minutes using a clear acrylic Y-shaped maze with arms that were 2" wide x 12" long x 12" high^{40,48}. The maze was placed on a table in a dimly lit room and spatial cues were displayed on walls around the table. Mice were placed in a randomized start arm and video tracking software was used to monitor arm entries (ANY-maze, Stoelting Co., IL, USA). An arm entry was called when the mouse's entire body, including the two back feet, entered the arm. The sequence and total number of arms entered was recorded, and the percentage of successful alternations was calculated as follows: number of alternations/maximum possible alternations (total number of arms entered- 2) x 100. Mice were evaluated for performance on this task longitudinally every two months until their specified harvest age (6m or 14m). AD-BXD and Ntg-BXD strain/age/sex specific group averages were generated for further downstream analyses.

2.4.4 Contextual fear conditioning

After 3 days of habituation to transport and to the testing room, mice underwent contextual fear conditioning (CFC) as previously described⁶⁴. On the first day of CFC training, mice were placed in a chamber and four 0.9mA 1 second (s) foot shocks were delivered after a baseline period. Over the period of 700s four foot shocks were delivered, and the post-shock intervals were defined as the 40s following the offset of each foot shock. The slope of the line formed when the percentage of time spent freezing following each shock was calculated as a function of each interval was used as a measure for contextual fear acquisition (CFA), and short-term memory. Twenty-four hours after training, mice were placed back into the training chamber and the percentage of time spent freezing throughout the entire 600s test was measured as an index of contextual fear memory (CFM), and long-term memory. AD-BXD and Ntg-BXD strain/age/sex specific group averages were generated for further downstream analyses.

2.4.5 RNA sequencing

Initial RNA sequencing (RNAseq) of hippocampus from the AD-BXD panel [6 months, n = 71 (47 females/24 males) and 14 months, n = 86 (45 females/41 males)], and Ntg-BXD counterparts [6 months, n = 31 (17 females/14 males) and 14 months, n = 33 (17 females/16 males)] has been previously reported ((Neuner et al., 2019a) GEO accession number GSE101144; (Neuner et al., 2019b) GEO accession number GSE119215). Here, we expand upon this dataset to include RNA sequencing data from an additional 71 Ntg-BXD mice, 27 of which were 6 months of age (22 females/5 males) and 44 of which were 14 months of age (28 females/16 males, GEO accession number GSE119408). RNA-seq was performed as previously described⁴⁸. Briefly, all samples were isolated using the Qiagen RNeasy Mini kit, libraries were prepared using Truseq Stranded mRNA Sample Preparation Kit (Illumina Inc), and sequenced by 75bp

sequencing on an Illumina HiSeq2500. Because sequencing was performed on mice harboring alleles for C57BL/6J (B6) and DBA2/J (D2), the GBRS/EMASE pipeline²⁷⁶ developed by the Churchill group at The Jackson Laboratory was used in order to align reads to the correct allele, allowing for quantification of both total reads for a gene and reads assigned to the B6 or D2 allele. To account for the expression of the 5XFAD transgene, RNA-seq reads were additionally aligned to the mutated human *APP* and *PSEN1* sequences. Genes were filtered to require an average of at least 1 transcript per million (TPM) in 50% of samples. RNA data was batch corrected using ComBat^{277,278}, and biological replicates were averaged together for downstream analyses. During batch correction, two samples (S3 and S285) were included in data upload, but dropped in final analyses as outliers before strain averaging. For final downstream analysis, individual samples belonging to the same BXD strain/sex/age/mutation carrier group were averaged to generate 156 distinct groups.

2.4.6 Formation of gene co-expression modules

Consensus clustering was employed on gene transcripts per million (TPM) values from the RNA-seq results using a novel algorithm⁷⁰ to identify groups of co-expressed genes or 'modules' that represented genes that were commonly regulated together across the examined mouse population. The consensus clustering algorithm, SpeakEasy, was used to identify 31 discrete transcriptional modules of co-expressed genes that ranged in size between 4 to 1419 gene members. 28 of the 31 modules contained more than 29 gene members, and could be assigned for >99% of the genes analyzed for co-expression. Therefore, three modules with fewer than 30 genes were excluded from downstream analysis, and contained <1% of total genes used for co-expression. This is consistent with previous methods for exclusion of modules with small membership⁷⁸.

2.4.7 Module functional enrichment

Gene Ontology (GO) functional enrichment analysis was performed on the genes within each module using the clusterprofiler package in R. GO categories with a hypergeometric enrichment adjusted $p < 0.05$ (adjusted for multiple comparisons using the Benjamini Hochberg correction) were considered to be significantly enriched within that corresponding module. Modules were named using their corresponding number as well as the top most significantly enriched GO category.

2.4.8 Module cell-type enrichment

Genes in each module were evaluated for cell-type enrichment from a previous study using a mineable set of genes obtained from RNA-seq of sorted cells from the adult mouse cerebral cortex²¹³. The cell-type assignment for each gene was determined by identifying the max Fragments Per Kilobase of transcript per Million mapped reads (FPKM) for each gene in one of the seven cell populations included in the RNA-seq dataset. Each module was evaluated for enrichment using hypergeometric tests to examine the overlap of module genes for genes in each cell type. The list of 12,978 genes downloaded from brainrnaseq.org was used as the background set, and module genes that had at least one cell-type assignment were used. A Benjamini Hochberg correction for multiple comparisons was performed using the `p.adjust()` function in R to obtain adjusted p-values of significant overlap from these tests.

2.4.9 Identification of module-trait relationships

Pairwise correlation analysis was employed to test the relationships between module expression and cognitive measures. First, the standardized average expression for each

module was calculated across all 156 strain/sex/age/mutation carrier group measures (setting average = 0, standard deviation = 1). To test the relationship of modules to cognition, we performed Spearman correlation analysis to identify the specific molecular networks that significantly related to individual differences in cognitive outcomes. Using the previously reported⁴⁸ cognitive measures for each corresponding strain/sex/age/mutation carrier grouping, Spearman correlations for module average expression to measures for working memory (assessed using the Y-maze task), short-term memory, and long-term memory (assessed using CFC) were calculated. We chose the Spearman correlation approach over Pearson correlations because when we tested for normality of cognitive outcomes and module expression across the population, while cognitive outcomes were normally distributed (**Figure 2.15**), module expression was not. Modules that were significantly correlated (Benjamini Hochberg adjusted $p < 0.05$) with at least one of these three traits were selected for inclusion of downstream Bayesian direct acyclic graph analysis.

2.4.10 ANOVA of module expression on binary-coded traits

Possible sources of variation in module expression were evaluated using a Type 2 ANOVA as a function of mutation carrier + age + sex (where mutation carrier denotes the presence/absence of the 5XFAD transgene) (**Figure 2.3, Table 2.1**). All p-values were adjusted using the Benjamini Hochberg correction for multiple comparisons.

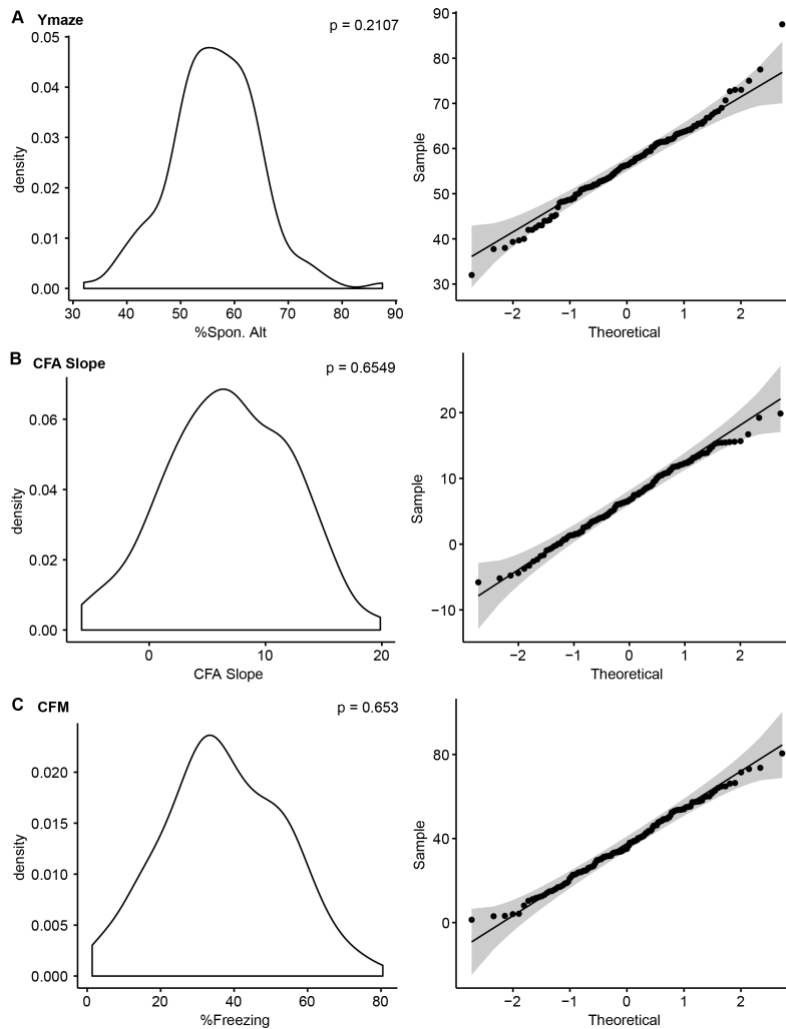


Figure 2.15: Tests of normality for AD-BXD and Ntg-BXD cognitive outcomes. A-C) Histograms (right) and q-q plots (left) depicting distribution and normality tests for cognitive outcomes for working memory (A), short-term memory (B) and long-term memory (C) outcomes for AD/Ntg-BXD population. The p-value depicted in the upper-right corner is result of Shapiro-Wilk test of normality for each cognitive outcome.

2.4.11 Calculation of variance explained

Effect size was calculated by generating a linear model using the `lm()` function in R, where formula = dependent ~ independent. In this formula, dependent variables corresponded to the cognitive measure for working memory (percent spontaneous alternations), short-term memory (CFA slope), or long-term memory (percent freezing

during CFC testing, CFM), and the independent variable was the standardized average expression for a specific module. A Type 2 ANOVA was performed, and the variance explained was obtained by taking the sum of squares for module expression and dividing by the total sum of squares for each model, which also corresponded to the multiple R^2 for each linear model. This analysis was performed for each module that was significantly correlated to working, short-term, and long-term memory measures. To determine the variance explained in AD-BXD individuals when amount of amyloid was added into the model, we first used a Type 3 ANOVA to test for significant interactions between module expression and amyloid amount. After identifying only one significant module*amyloid interaction (module 4*amyloid for Y-maze %SA), we continued with an additive Type 2 ANOVA model. For this model, the same protocol was employed as before in which variance explained was calculated using individual sum of squares for each independent variable divided by the total sum of squares (including residual sum of squares). Likelihood-ratio (LRT) tests were performed to determine if the addition of cortical A β 1-42 into the linear model significantly improved the fit. In this calculation, model 1: cognitive outcome ~ module expression was tested against model 2: cognitive outcome ~ module expression + cortical A β 1-42 ELISA to obtain a p-value which reflected if the two models were significantly different from each other. LRT tests with $p < 0.05$ were interpreted to have a better fit with the addition of cortical A β 1-42 than with module expression alone. Results of ANOVA and LRT tests are depicted in **Table 2.4**.

2.4.12 Module-trait network

The module-trait network was constructed using the pre-defined relationships of modules that significantly correlated with at least one cognitive outcome, as well as genetic information provided by Quantitative trait locus (QTL) mapping of module eigengenes. This module-trait network of mixed node times illustrated conditional

dependencies among all variables (module expression, cognitive measures), and utilized genomic priors (genetic regions associated with module eigengenes) to help determine the orientation of edges in the resulting network^{78,212}.

2.4.13 Module QTL mapping

Quantitative trait locus (QTL) mapping was employed using the eigengene for each module significantly correlated with cognitive outcomes. Genotypes for the BXD strains used in this study were obtained from GeneNetwork.org²⁷⁹, and module eigengenes (ME) were generated by calculating the first principal component (PC1) of module expression. The BXD genotypes and module eigengenes were employed for downstream QTL mapping in *r/qtl*²⁸⁰. ANOVA of module expression (**Figure 2.3**) were used to establish age sex, and/or genotype as additive covariates for each module. Covariates were used as additive, and 1000 permutations were used to determine statistical significance.

2.4.14 Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) was performed to complement the initial GO enrichment for gene co-expression modules. Specifically, the top enriched canonical pathways (by enrichment p-value) in the IPA Canonical Pathway Analysis report were analyzed to gain a more specific understanding of enriched pathways in modules.

2.4.15 Enzyme-linked immunosorbent assay (ELISA)

Data for beta-amyloid 1-42 (A β 42) quantification was used from ELISA arrays performed and described in our previously published study⁴⁸. Briefly, A β 1-42 levels were quantified from sections of temporal cortex [6 m n = 72 mice (46 female/46 male) across

22 AD-BXD strains, 14 m n = 82 mice (43 female/33 male) across 21 AD-BXD strains]. Tissue was homogenized in 1X PBS + 1% Triton X-100 using the TissueLyser II system (QIAGEN) and sonicated 2x10s on low power. Protein concentration was determined using a NanoDrop 2000 UV-Vis Spectrophotometer (ThermoScientific, USA). Brain homogenates (10 mg/ml) were extracted in a final concentration of 5M GuHCl overnight at 4°C. Samples were then diluted appropriately and run in duplicate on A β 1-42-specific sandwich colorimetric ELISAs according to the manufacturer's protocol (Cat# 298-92401, Wako Chemicals, Richmond, VA). Optical densities at 450 nm were read on a Biotek plate reader (BioTek, USA) and A β 1-42 concentration was determined by comparison with A β 1-42 standard curves. Only readings in the linear range of the standard curve were included in analysis. Duplicates were averaged to determine concentration of A β 1-42 in each sample. Finally, A β 1-42 concentrations were normalized to total protein concentration and are reported as nanograms of A β 1-42 per milligrams of total protein.

2.4.16 Mouse-to-human module comparisons

Gene co-expression modules formed by employing SpeakEasy clustering on bulk RNA-seq from human dorsolateral pre-frontal cortex (DL-PFC) tissue were obtained from a previously published Religious Orders Study and Memory and Aging Project (ROSMAP) dataset⁷⁸, as well as co-expression modules from multiple human brain regions from the Accelerating Medicine Partnership- Alzheimer's Disease (AMP-AD) consortium⁷⁷. In the Mostafavi study, there were 47 modules formed with at least 20 gene members, and modules were related to human cognitive outcomes using a correlation approach similar to what was performed in this current study. In the Logsdon study, there were 30 primary modules generated across 7 brain regions and 3 studies compiled from a meta-analysis of human aged and AD brains. Human genes from these

modules were converted to orthologous mouse genes using the biomaRt() package in R. Hypergeometric tests were employed between each human and mouse module to examine the degree of conservation between the two species. The background gene list used was the total number of unique genes that could be found in any human or mouse module. The Benjamini-Hochberg correction for multiple comparisons was performed to obtain adjusted p-value using the p.adjust() function in R. All human and mouse modules that passed the preliminary filtering step (mouse modules >29 genes, Mostafavi human modules >20 genes, all Logsdon modules) were examined for this analysis.

2.4.17 Statistical analysis and software

R software version 3.6.3 was used for data analysis. Code for SpeakEasy clustering was obtained from an online repository (<https://www.cs.rpi.edu/~szymansk/SpeakEasy/index.html>)⁷⁰, and run in Matlab version 2018a. Statistical tests included ANOVA, hypergeometric tests for overlap, Spearman correlations, and Benjamini Hochberg corrections for multiple testing. All scripts used for these analyses have been posted for open access to GitHub (Heuer_2020) and data files on Synapse.org (10.7303/syn17016211). Code for Bayesian modeling was obtained from an online repository at Synapse.org (10.7303/syn2910187)²¹².

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2.6 Author contributions

S.E.H. and C.C.K. conceived of experiments. S.E.H., S.M.N., C.G., and C.C.K. designed and implemented experiments and analysis. S.E.H., N.H. and V.M.P. interpreted, designed, and implemented statistical analysis. S.M.N. conducted behavioral experiments, ELISA assays, and extracted RNA for sequencing. C.C.K., C.G., V.M.P., K.M.S.O., R.W.W., S.M.N, and N.H. assisted S.E.H. in designing experiments and interpretation of results, as well as preparation of manuscript. All authors reviewed and approved of the final manuscript.

Chapter 3: Genetic context drives age-related disparities in synaptic maintenance and structure across cortical and hippocampal neuronal circuits²

²**Sarah E. Heuer**, Emily W. Nickerson, Gareth R. Howell, Erik B. Bloss. Submitted to *Aging Cell*, 8/3/2023.

3.1 Introduction

Synapses are the fundamental computational subunits of the brain, and the building blocks of neuronal circuits that vary in complexity based on brain region and mammalian organism^{148,149}. Synapses enable cell-type specific forms of communication and are often targeted to defined spatial subcellular locations on postsynaptic cell types²⁸¹, which constrains neural circuit operations^{150,151}. Synapses act as plastic, tunable biochemical and electrical signaling compartments, a feature that allows internal states to shape neural circuit function^{282,283}, and may be the primary substrate in the nervous system mediating learning and memory recall^{92,284}.

Individual excitatory synapses on many neuronal cell types take the form of small protrusions along the dendrites (i.e., dendritic spines¹⁶¹), which can be visualized by multiple imaging methods^{92,154,155}. Spine structure reflects the functional strength of each connection: spine head size correlates with synapse strength^{92,155}, and the morphology of the spine neck determines the degree of electrical and biochemical filtering that occurs between the synapse and the parent dendritic cable^{157,159}. During normal aging, mammalian organisms experience progressive, yet variable, cognitive decline, even in the absence of a disease, which corresponds to a loss in synaptic plasticity²⁸⁵. Relative to other synapses, spine synapses seem to be the primary type that are lost or altered during aging, and these changes in the aging brain appear circuit specific^{168,175}. For example, the density of spine synapses decrease over time on cortical neurons in the frontal but not visual cortex, and decrease on CA3 pyramidal neurons but not on neighboring neurons in CA1^{168,286,287}. The loss of synapse number and the changes in synaptic structure on frontal cortical neurons correlate with progressive decreases in cognitive function with age^{176,288,289}, yet reports of the degree to which synaptic loss occurs suggests significant heterogeneity^{163,168,172,173}. The causal factors that underlie this variability could be what determines successful versus unsuccessful neural

aging^{72,168,174}, and there is significant interest in understanding how genetic components and environmental influences coordinately shape the trajectory of age-related cognitive changes and synaptic connectivity.

Non-human primates (NHPs)^{171,176,290} and rodents (rats^{163,291,292} and mice²⁹³) are commonly used model systems to study the effect of aging on synapses. Although NHPs are the most translatable model for relating age-related neural circuit changes to cognitive decline^{165,176}, they have long lifespans (>30 years), require complex housing systems, and are not amenable to genetic-based experimentation at scale. However, to a large extent, data from rodent models recapitulate the magnitude of age-related synaptic loss in vulnerable brain regions (including the prefrontal cortex) that has been observed in NHPs^{163,170,173}. Studies in mice have shown loss in synaptic plasticity and number during aging and in age-related diseases^{52,177}. Unlike NHP experiments, which necessarily use genetically diverse non-inbred subjects, the work in mice has largely been performed using the inbred C57BL/6J (B6) mouse strain. The lack of genetic diversity limits translatability of mouse work to humans, and led us to evaluate the potential of incorporating genetically distinct wild-derived inbred mouse strains in our own work⁵¹. Our previous results show wild-derived strains exhibit diverse cognitive and behavioral phenotypes during aging and when carrying Alzheimer's disease (AD)-linked transgenes, suggesting genetic background strongly influences neuronal circuitry and function^{53,294}. In particular, the PWK/PhJ (PWK) mouse strain did not show amyloid-dependent cognitive decline or synaptic loss, suggesting PWK is an ideal strain to study resilience to AD²⁹⁵. However, the neuronal circuits vulnerable in aging are different than those vulnerable to AD, and it remains unknown whether PWK is also resistant to normal age-related synaptic loss.

To determine the effect of genetic context on age-related synapse dynamics (and how they might relate to their observed cognitive susceptibility/resilience^{53,296}), we

examined aging-related synapse loss in the traditionally studied B6 mouse strain and the wild-derived PWK. PWK mice differ from B6 by approximately 20 million single nucleotide polymorphisms (SNPs) and structural variants⁵¹. To target our analyses to specific projection neurons, we leveraged retrograde viral strategies to label two circuits known to have different vulnerabilities to aging or AD: PFC-projecting neurons to nucleus reuniens (RE; PFC-to-RE), and CA1-projecting neurons to PFC (CA1-to-PFC)¹⁷⁵. Spine synapses on CA1-to-PFC neurons showed marked stability throughout aging in both B6 and PWK mice. In contrast, PFC-to-RE neurons were susceptible to age-related spine loss in B6 but not PWK. Further, there were morphological changes suggestive of synapse strengthening in PWK but not B6 mice. Collectively, these results provide evidence that PWK are a model of successful synaptic aging.

3.2 Results

3.2.1 Labeling strategies to visualize synapses from two neural circuits in genetically distinct mice.

Our previous studies suggest PWK are a model of cognitive, neuronal and synaptic resilience to A β pathology^{53,295}. However, since age is the leading risk factor for developing AD, we tested the prediction that PWK would also be a model of successful synaptic aging when compared to traditionally used inbred B6 mice. We leveraged previously established viral approaches^{166,167} using two different fluorophores to label excitatory projection neurons forming two arms of a connected hippocampal-cortico-thalamic loop important for spatial working memory tasks²⁹⁷. Each set of projection neurons expressed either EGFP or tdTomato across cohorts of female B6 and PWK mice ranging from 4-29 months of age (see **Methods, Table 3.1**). Three unilateral intracranial injection sites were targeted to deliver recombinant adenoassociated virus

(AAV, see **Methods**) to drive EGFP in PFC neurons that project to RE (PFC-to-RE) and tdTomato expression in CA1 neurons that project to PFC (CA1-to-PFC). After allowing 3-4 weeks for proper retrograde movement and fluorophore expression each mouse was perfused, coronal slices were made with a vibratome, slices containing EGFP+ or tdTomato+ neurons were imaged, and dendritic spine density and morphologies were analyzed (**Figure 3.1A**). Three age groups were assessed: young (4-7 months of age), middle-aged (11-17 months of age) and aged (22-30 months of age) groups. Neurons making up the two projection circuits in B6 and PWK mice were labeled with equivalent efficiency, suggesting a similar overall density of neurons comprising these pathways from the compared strains (**Figure 3.1B-C**).

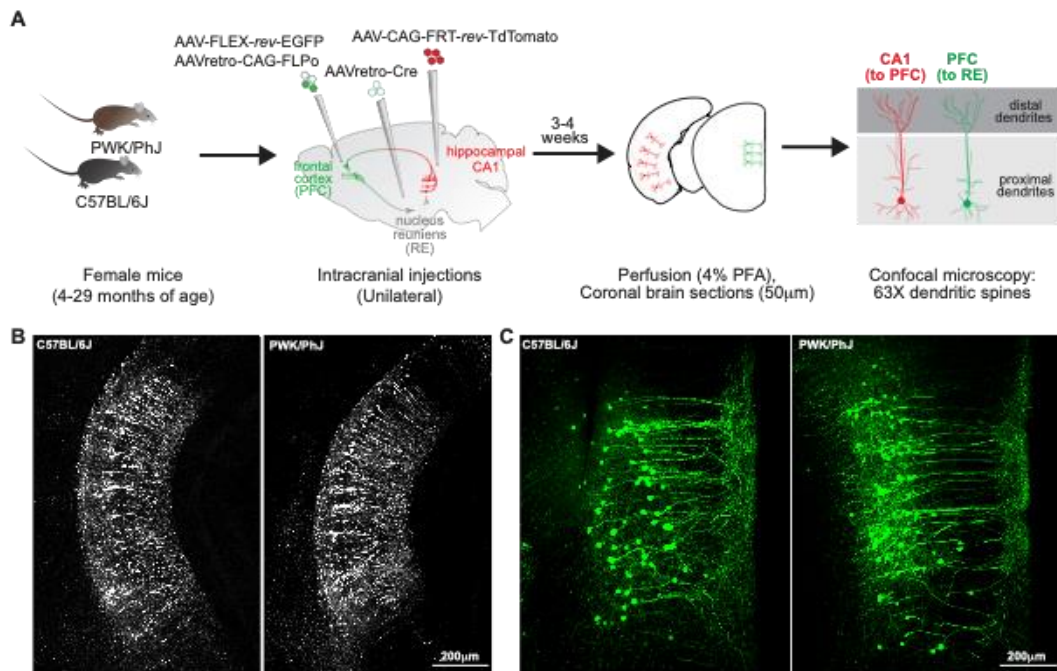


Figure 3.1: Viral labeling of PFC-to-RE and CA1-to-PFC neurons across C57BL/6J and PWK/PhJ mice.

(A) Experimental outline (see **Methods** for additional details). (B-C) Example 10X images of tdTomato+ CA1 neurons (B) from B6 (left) and PWK (right) demonstrating consistent CA1-to-PFC projection circuit labeling, and EGFP+ PFC neurons (C) from B6 (left) and PWK (right) demonstrating consistent PFC-to-RE projection circuit labeling. Summary metadata for individual mice used in this study reported in **Table 3.1**.

3.2.2 Aging spines on proximal CA1 dendrites exhibit stable densities but altered morphologies in PWK but not B6 mice

The CA1 region of the hippocampus is well-studied on account of its anatomy, physiology, role in episodic memory, and vulnerability to both age- and AD-related pathological insults⁷². While generally thought to be relatively resistant to normal age-related synaptic loss^{298–300}, it is unclear if age-related structural changes occur on specific CA1 projections, and if they are modulated in subjects from genetic contexts that exhibit resistance to cognitive aging. The majority of excitatory inputs onto CA1 pyramidal cells are made onto the proximal apical oblique and basal branches^{301,302} which both receive inputs from hippocampal area CA3³⁰³. Therefore, we first analyzed dendritic spine synapses on branches from this proximal compartment specifically on CA1-to-PFC neurons (**Figure 3.2A**). Consistent with the prediction from previous reports^{175,298}, we observed no significant age-related changes in proximal spine density in either B6 or PWK mice (**Figure 3.2B-C**).

Dendritic spines alter their morphologies in response to repeated artificial synaptic stimulation^{282,284}, under conditions of homeostasis^{283,304}, and in reaction to disease pathologies and during aging^{305,306}. Spine head morphology is a reliable predictor of synaptic stability and strength^{92,155}, so we analyzed the maximum head diameter of each reconstructed spine. Our analysis found no overt age-related changes in B6 mice, but the same analysis in PWK mice revealed a subtle yet significant age-related shift to spines with smaller head diameters (**Figure 3.2D**). This effect was evident when we examined the densities of spines across the smallest (Q1) and largest (Q4) quartiles of head diameters (**Figure 3.3B**).

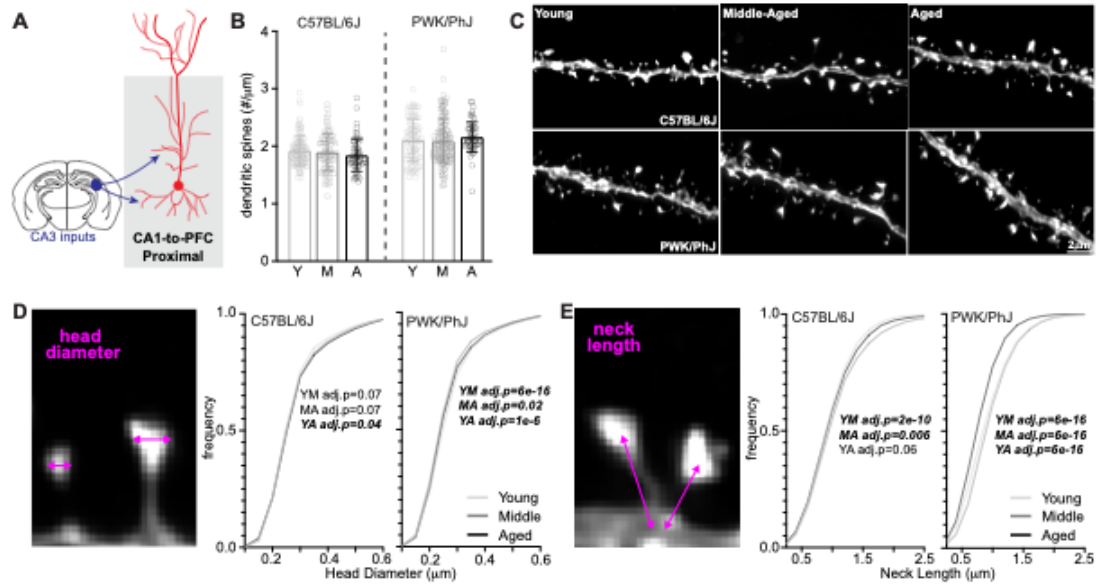


Figure 3.2: CA1-to-PFC proximal dendrites are resistant to synaptic density changes across both strains, but PWK/PhJ spines change morphologies. **(A)** Example schematic of tdTomato+ proximal CA1-to-PFC dendrites analyzed here and projection input origins in hippocampal area CA3. **(B)** Proximal CA1-to-PFC dendrite spine densities (spines/μm) comparing young (Y), middle (M) and aged (A) within each strain. Data points represent individual branches (n=20/mouse); error bars are $\pm$ SD; one-way ANOVA identified no significant effects within B6 or PWK. **(C)** Representative 63X images of proximal CA1-to-PFC dendrites from each strain/age group. **(D)** CA1-to-PFC proximal spine head diameter example image (left) and cumulative distributions from B6 (middle) and PWK (right), across age groups. Kolmogorov-Smirnov tests were used to evaluate statistical significance (Bonferroni adj. $p < 0.05$) of pairwise comparisons: young vs middle (YM), middle vs aged (MA), and young vs. aged (YA), and p-values reported on each graph. Data points are representative of measures from individual spines. **(E)** Same as **(D)** for CA1-to-PFC proximal spine neck length.

The morphology of dendritic spine necks (e.g. neck width and length) are similarly plastic¹⁵⁹ and define the compartmentalization of spines much more critically than spine head size^{157,159,160,307}. Dendritic spine necks act as strong electrical resistors, filtering currents from the synapse to the parent dendrite branch¹⁶⁰. Neck also contain receptors for neuromodulatory systems that regulate local excitability³⁰⁸. Spine necks are often below 50 nm³⁰² making them hard to resolve with light microscopy techniques, thus we analyzed putative spine neck lengths to determine whether this aspect of spine neck

morphology is readily modified during aging. Neck lengths in B6 mice were slightly lengthened with age, complementing the stability observed in spine heads. In contrast, PWK spine neck lengths showed a biphasic response, first increasing from young to middle-aged, then decreasing significantly in aged mice compared to the younger groups (Figure 3.2E, Figure 3.3C).

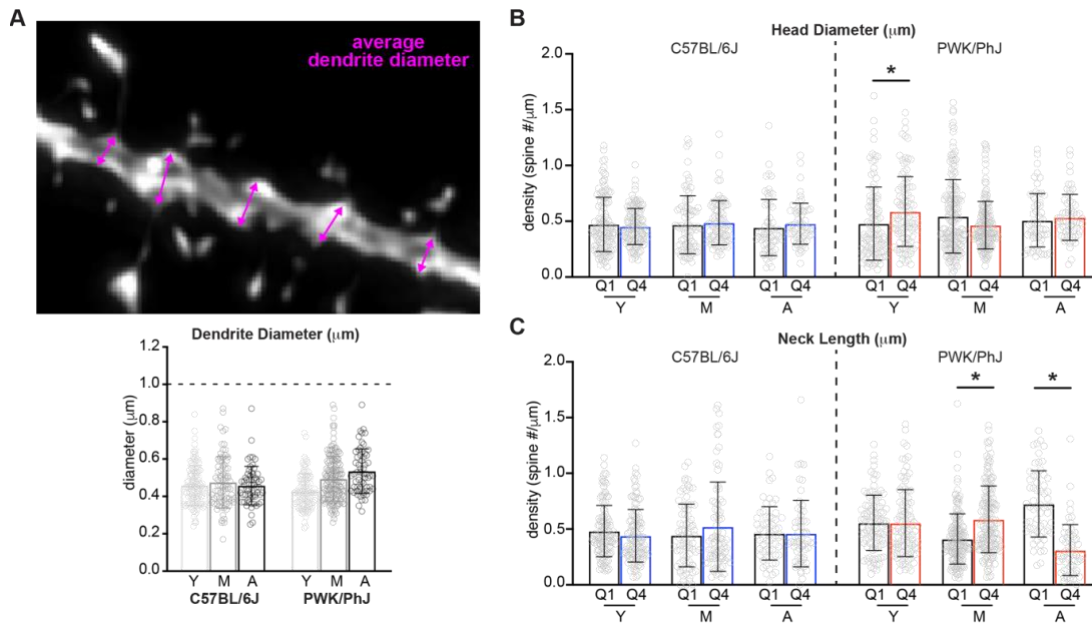


Figure 3.3: Additional morphological analyses for proximal CA1-to-PFC spines and dendrites, corresponding to Figure 3.2.

(A) CA1-to-PFC proximal average dendrite diameter example image (top) and quantification of average diameter for each analyzed dendrite (bottom). Horizontal dashed line represents that dendrites sampled were under 1 μm in diameter, corresponding to uniformly thin branches. Data points represent individual branches (n=20/mouse). Groups measured include young (Y), middle-aged (M) and aged (A). (B) Quartile-based analyses of CA1-to-PFC proximal spine head diameters. All proximal CA1-to-PFC spines within each strain were divided into quartiles based on head diameter (μm). The smallest spines assigned to the first quartile (Q1) and the largest spines assigned to the fourth quartile (Q4) were identified and reassigned back to originating dendrite. Spine densities (spines/μm) for Q1 and Q4 spines were calculated separately. Data points represent individual branches. Nonparametric two-tailed t-tests were performed to compare Q1 to Q4 within each strain/age group to identify significant (*=p<0.05) shifts in size. (C) Same as (B) for CA1-to-PFC proximal spine neck length.

3.2.3 Spine changes on distal CA1 dendrites mirror those found on proximal compartments

Distal tuft dendrites in CA1 pyramidal cells are thought to be the most vulnerable to AD-related synaptic changes due to their afferent inputs originating in the EC (**Figure 3.4A**)³⁰⁹, where associated neuropathology is observed very early in the disease process^{310,311}. Synapses on distal dendrites are understudied relative to those on more proximal compartments due to limitations in traditional techniques used to evaluate plasticity at remote locations relative to the soma. Consistent with our results from the proximal dendrites, we observed no significant age-related changes to distal tuft spine densities in B6 or PWK mice (**Figure 3.4B-C**). Like spines from proximal branches, we observed few age-related changes in head diameter and neck length in spines from B6 distal branches (**Figure 3.4D-E, Figure 3.5B-C**). However, spine head diameters were larger in aged PWK mice compared to young and middle-aged cohorts (**Figure 3.4D, Figure 3.5B**), and spine neck lengths were significantly shorter in aged compared to young and middle-aged mice (**Figure 3.4E, Figure 3.5C**). While subtle in nature, the larger differences observed between young and aged cohorts would suggest functional changes occurring to the excitatory neurons affecting synaptic signaling and filtering to the parent dendrite.

Collectively, these results show spine densities on proximal and distal dendrites from CA1-to-PFC pyramidal cells do not change with age in both B6 and PWK mice. Furthermore, B6 spines remained morphologically stable whereas PWK spines exhibited changes indicative of increased excitability at the synapse and to the parent dendritic cable, which support the notion that these adaptations could play a role in maintaining information processing in the aging hippocampus.

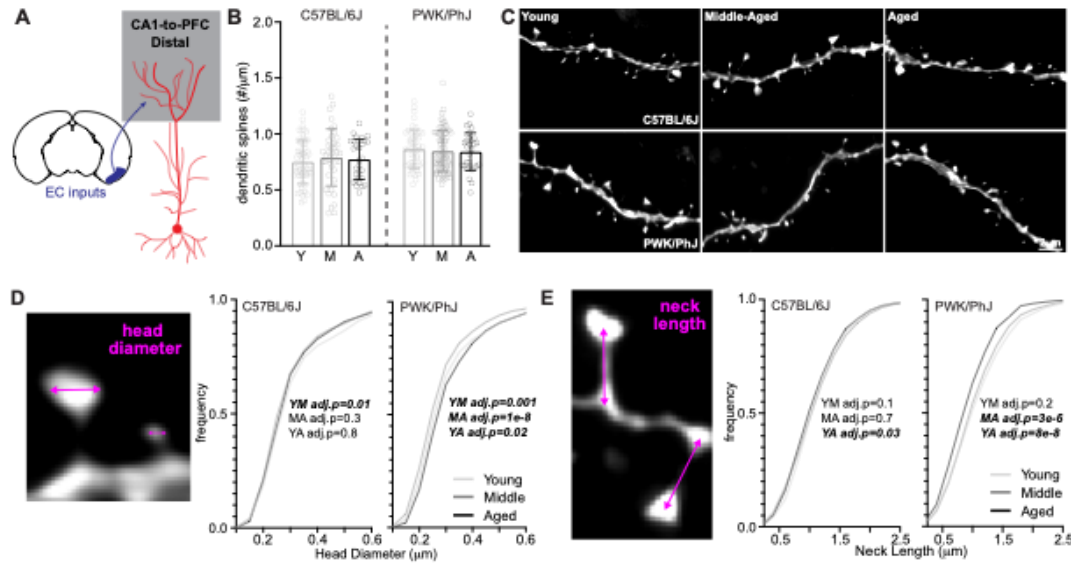


Figure 3.4: CA1-to-PFC distal tuft dendrites do not lose synapses but are morphologically altered in PWK/PhJ mice.

(A) Example schematic of tdTomato+ distal tuft CA1-to-PFC dendrites analyzed here and projection input origins in entorhinal cortex (EC). (B) Distal tuft CA1-to-PFC dendrite spine densities (spines/ μ m) comparing young (Y), middle (M) and aged (A) within each strain. Data points represent individual branches (n=10/mouse); error bars are $\pm$ SD; one-way ANOVA identified no significant effects within B6 or PWK. (C) Representative 63X images of distal tuft CA1-to-PFC dendrites from each strain/age group. (D) CA1-to-PFC distal tuft spine head diameter example image (left) and cumulative distributions from B6 (middle) and PWK (right), across age groups. Kolmogorov-Smirnov tests were used to evaluate statistical significance (Bonferroni adj. $p < 0.05$) of pairwise comparisons: young vs middle (YM), middle vs aged (MA), and young vs. aged (YA), and p-values reported on each graph. Data points are representative of measures from individual spines. (E) Same as (D) for CA1-to-PFC distal tuft spine neck length.

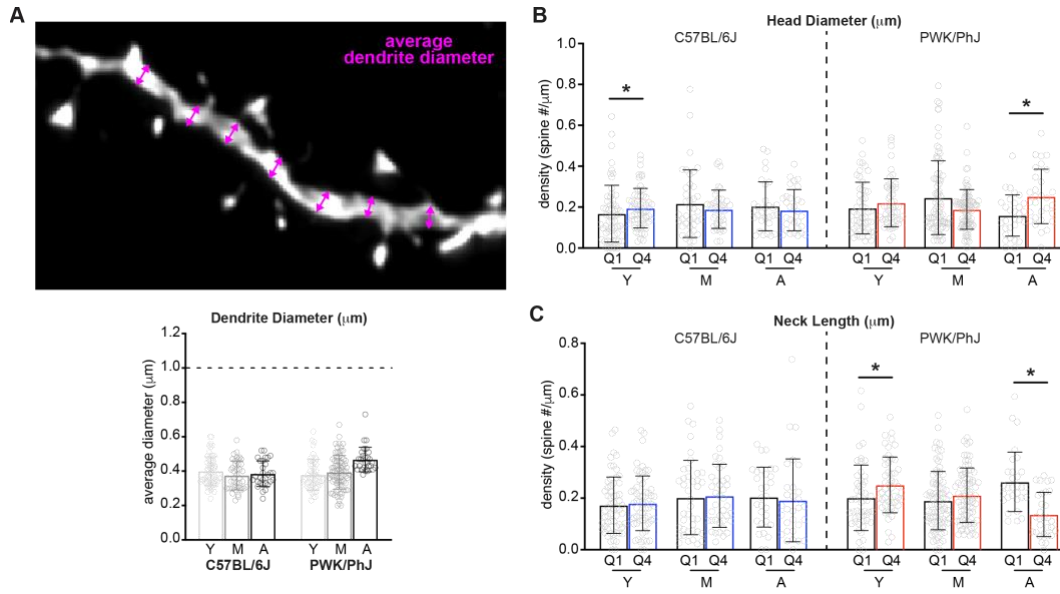


Figure 3.5: Additional morphological analyses for distal tuft CA1-to-PFC spines and dendrites, corresponding to Figure 3.4.

(A) CA1-to-PFC distal average dendrite diameter example image (top) and quantification of average diameter for each analyzed dendrite (bottom). Horizontal dashed line represents that dendrites sampled were under 1 μm in diameter, corresponding to uniformly thin branches. Data points represent individual branches (n=10/mouse). Groups measured include young (Y), middle-aged (M) and aged (A). (B) Quartile-based analyses of distal tuft CA1-to-PFC spine head diameters. All distal tuft CA1-to-PFC spines within each strain were divided into quartiles based on head diameter (μm). The smallest spines assigned to the first quartile (Q1) and the largest spines assigned to the fourth quartile (Q4) were identified and reassigned back to originating dendrite. Spine densities (spines/ μm) for Q1 and Q4 spines were calculated separately. Data points represent individual branches. Nonparametric two-tailed t-tests were performed to compare Q1 to Q4 within each strain/age group to identify significant (*= $p < 0.05$) shifts in size. (C) Same as (B) for CA1-to-PFC distal tuft spine neck length.

3.2.4 Differential vulnerability to age-related synaptic changes on PFC-to-RE neurons across B6 and PWK mice

Previous studies in humans, NHPs, rats and mice report that pyramidal neurons in the prefrontal cortex are susceptible to synaptic loss with age^{163,176,289,290,312}. Moreover, preventing this degeneration could be key to promoting successful cognitive aging^{289,313,314}. To test the prediction that PWK mice are resistant to age-dependent synaptic loss in the PFC, we used AAVs to also label deep-layer neurons (in the exact mice described above) in the PFC that project to the RE (Figure 3.1) that appear to

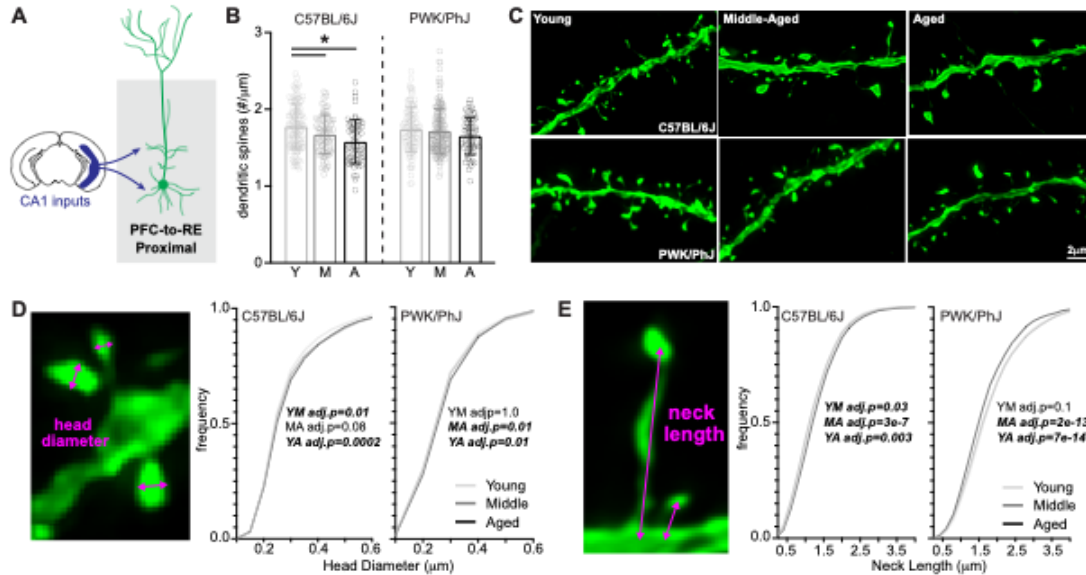


Figure 3.6: C57BL/6J mice are susceptible to age-related synaptic loss in proximal PFC-to-RE dendrites, with PWK/PhJ demonstrating dynamic morphological changes to increase synaptic strength.

(A) Example schematic of EGFP+ proximal PFC-to-RE dendrites analyzed here and projection input origins in hippocampal area CA1 **(B)** Proximal PFC-to-RE dendrite spine densities (spines/μm) comparing young (Y), middle-aged (M) and aged (A) within each strain. Data points represent individual branches (n=20/mouse); error bars are $\pm$ SD; one-way ANOVA identified significant effect within B6 but not PWK; asterisks denote significant (Bonferroni adjusted $p < 0.05$) comparisons identified using post-hoc analysis within B6. **(C)** Representative 63X images of proximal PFC-to-RE dendrites from each strain/age group. **(D)** PFC-to-RE proximal spine head diameter example image (left) and cumulative distributions from B6 (middle) and PWK (right), across age groups. Kolmogorov-Smirnov tests were used to evaluate statistical significance (Bonferroni adj. $p < 0.05$) of pairwise comparisons: young vs middle (YM), middle vs aged (MA), and young vs. aged (YA), and p-values reported on each graph. Data points are representative of measures from individual spines. **(E)** Same as **(D)** for PFC-to-RE proximal spine neck length.

mediate behavioral flexibility and working memory^{315–317}. Like the CA1-to-PFC neurons, PFC-to-RE neurons have a similar proximal-distal dendritic architecture, and each compartment receives a distinct set of inputs. Basal and apical oblique dendrites located in the proximal regions receive afferent inputs from CA1 (among other regions), whereas distal tuft receive a distinct set of inputs from the thalamus¹⁶⁷ (**Figure 3.6A**). Therefore, we analyzed the spines on proximal and distal dendrites separately.

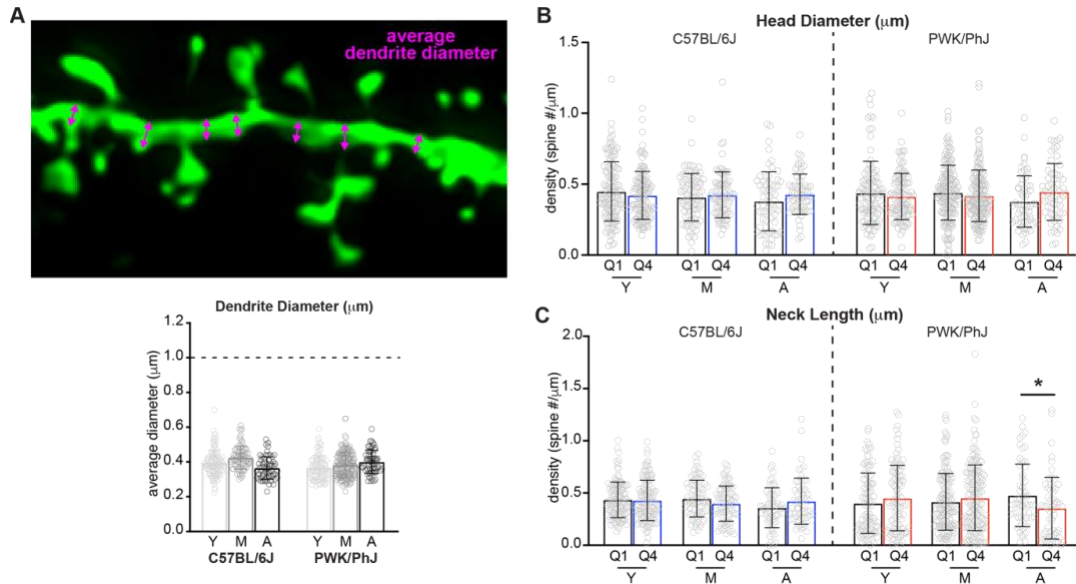


Figure 3.7: Additional morphological analyses for distal tuft PFC-to-RE spines and dendrites, corresponding to Figure 3.6.

(A) PFC-to-RE proximal average dendrite diameter example image (top) and quantification of average diameter for each analyzed dendrite (bottom). Horizontal dashed line represents that dendrites sampled were under 1 μm in diameter, corresponding to uniformly thin branches. Data points represent individual branches (n=20/mouse). Groups measured include young (Y), middle-aged (M) and aged (A). **(B)** Quartile-based analyses of proximal PFC-to-RE spine head diameters. All proximal PFC-to-RE spines within each strain were divided into quartiles based on head diameter (μm). The smallest spines assigned to the first quartile (Q1) and the largest spines assigned to the fourth quartile (Q4) were identified and reassigned back to originating dendrite. Spine densities (spines/μm) for Q1 and Q4 spines were calculated separately. Data points represent individual branches. Nonparametric two-tailed t-tests were performed to compare Q1 to Q4 within each strain/age group to identify significant (*= $p < 0.05$) shifts in size. **(C)** Same as **(B)** for PFC-to-RE proximal spine neck length.

We first examined spine density on proximal dendrites of PFC-to-RE neurons. Consistent with previous findings, B6 proximal dendritic spine density was significantly lower in middle-aged and aged compared to young mice³¹⁸. Interestingly, we found no differences in spine densities on proximal PFC dendrites across age cohorts in PWK mice (**Figure 3.6B-C**). When we examined spine morphologies, we found statistically significant changes in spine head diameter with age on both B6 and PWK proximal PFC dendrites, evidenced by the cumulative frequency analyses and by quartile analysis of the largest and smallest spines (**Figure 3.6D**, **Figure 3.7B**). In both B6 and PWK mice,

PFC proximal spine head diameters became subtly larger with aging (**Figure 3.6D**). Interestingly, spines on proximal dendrites from B6 mice showed minimal age-related changes in spine neck length. However, spines on proximal branches from PWK mice were significantly shortened with age, changes that should reduce neck-related filtering of synaptic currents, mimicking the same patterns found on aging CA1 dendrites from PWK mice (**Figure 3.6E, Figure 3.7C**).

3.2.5 Age-related dendritic spine loss and morphological remodeling on distal PFC dendrites differs between B6 and PWK mice

Distal tuft branches from PFC-to-RE projection neurons receive distinct afferent inputs compared to proximal branches, coming from the thalamus instead of CA1¹⁶⁷ (**Figure 3.8A**). Similar to the age-related changes found on proximal dendrites of PFC-to-RE neurons, distal dendritic spine densities were lower in middle-aged and aged compared to young B6 mice but did not change across age groups in PWK mice (**Figure 3.8B-C**).

Spine head diameters on distal branches of PFC-to-RE neurons from B6 mice showed a biphasic response, first increasing in size between young and middle-aged cohorts, then decreasing in size between middle-aged to aged groups. PWK distal PFC spine heads displayed the opposite pattern: decreasing in size between young and middle-aged groups, and then increasing in size between middle-aged and aged cohorts (**Figure 3.8D, Figure 3.9B**). Spine neck lengths on the distal dendrites of PFC-to-RE neurons from B6 mice slightly lengthened with age, while those from PWK decreased more dramatically with age (**Figure 3.8E**). These changes in spine neck lengths were observed in quartile analyses, with a significant gain of long spine necks in aged B6 mice, and an overt loss of long spine necks in aged PWK mice (**Figure 3.9C**). These results from distal PFC-to-RE dendrites mirrored those from proximal dendrites: B6

branches showed age-related synaptic loss while those from PWK did not. In contrast, PWK distal PFC spines showed spine neck remodeling while those from B6 were markedly more stable.

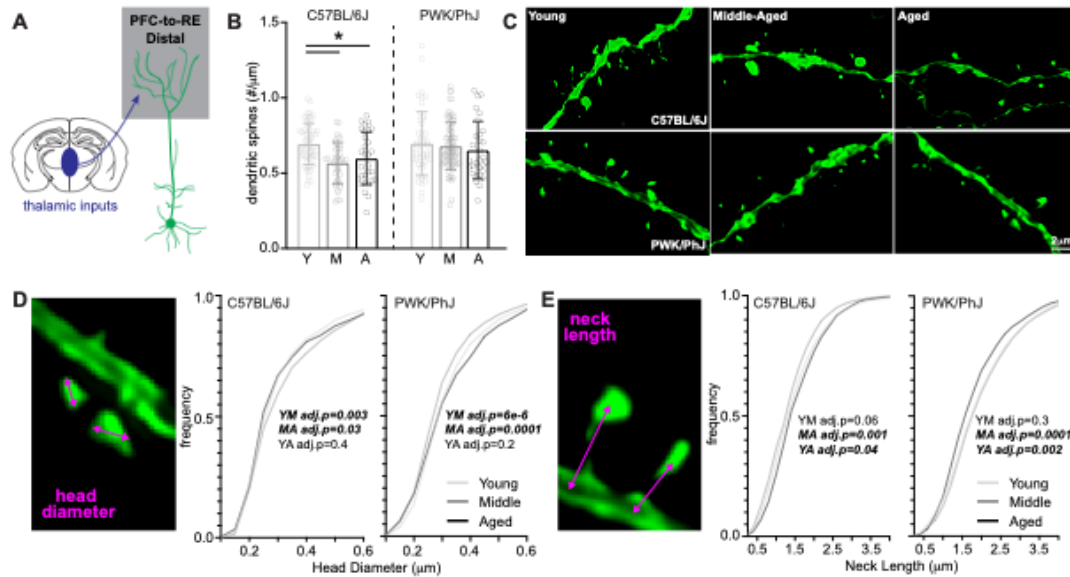
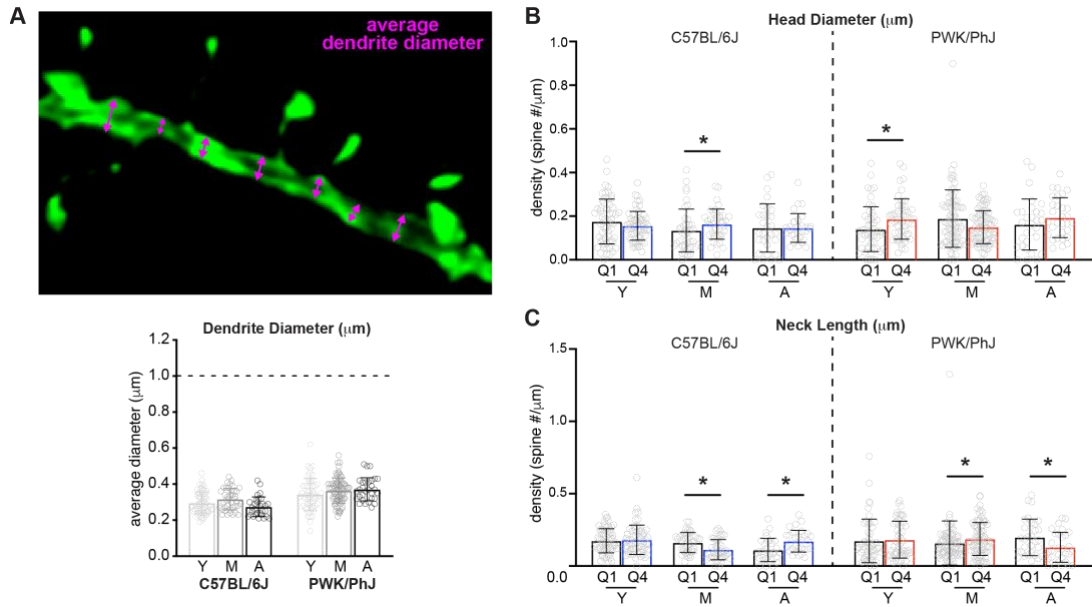


Figure 3.8: PFC-to-RE distal tuft dendrites exhibit age-induced synaptic loss in C57BL/6J mice but are morphology dynamic in PWK/PhJ mice.

(A) Example schematic of EGFP+ distal tuft PFC-to-RE dendrites analyzed here and thalamic projection input origins. (B) Distal tuft PFC-to-RE dendrite spine densities (spines/μm) comparing young (Y), middle (M) and aged (A) within each strain. Data points represent individual branches (n=10/mouse); error bars are $\pm$ SD; one-way ANOVA identified significant effect within B6 but not PWK; asterisks denote significant (Bonferroni adjusted $p < 0.05$) comparisons identified using post-hoc analysis within B6. (C) Representative 63X images of distal tuft PFC-to-RE dendrites from each strain/age group. (D) PFC-to-RE distal tuft spine head diameter example image (left) and cumulative distributions from B6 (middle) and PWK (right), across age groups. Kolmogorov-Smirnov tests were used to evaluate statistical significance (Bonferroni adj. $p < 0.05$) of pairwise comparisons: young vs middle (YM), middle vs aged (MA), and young vs. aged (YA), and p-values reported on each graph. Data points are representative of measures from individual spines. (E) Same as (D) for PFC-to-RE distal tuft spine neck length.

Collectively, these data support our hypothesis that, like resilience to AD-related A β pathology⁵³, PWK mice show resistance to age-related cortical synapse loss. This resistance was evident across both proximal and distal compartments and accompanied by specific forms of spine head and neck remodeling. Decreases in spine neck length should serve to lower the spine neck resistor and effectively increase the efficiency by

which current generated at the synapse reaches the parent dendrite. Given the growing links between dendritic mechanisms of integration and the plasticity that mediates learning and memory^{319–322}, the putative adaptive spine responses could serve as mechanisms to promote successful maintenance of neuronal health and cognition during aging.



3.3 Discussion

Here, we asked whether the effects of aging on synapse number and structure in two specific neural circuits was equivalent across two distinct strains of mice. We chose to investigate B6 and PWK strains as our past work has identified them as susceptible or resilient to AD-related cognitive and synaptic deficits, respectively^{53,295,296}. To rigorously compare across defined neural circuits, we used multisite viral injections to label CA1-to-PFC and PFC-to-RE neurons and systematically sampled ~90,000 spines in proximal and distal dendritic compartments across multiple ages (ranging from 5-30 months of age). We found that PFC-projecting CA1 pyramidal cells from both strains maintain their dendritic spine synaptic densities with age, consistent with a body of work examining randomly labeled neurons or synapses in aging NHPs, rats and B6 mice^{72,168,171,286,298}. Conversely, we observed a differential pattern of synapse loss on RE-projecting PFC neurons between B6 and PWK mice, with synapse loss restricted to B6 but not PWK mice. Spine reconstructions suggested that spines from B6 showed either stability or patterns of remodeling putatively associated with decreased synaptic efficacy, whereas PWK spines tended to show morphological changes consistent with increased synaptic efficacy during aging (**Tables 3.1-3.2**). These results suggest that previously observed cognitive characteristics from these strains⁵³, including PWK resistance to aging-related decline in cognition or resilience to AD-related neuropathology⁵³ and differential microglia activation⁵⁴ may be due in part to an adaptive forms of synaptic plasticity that maintain connectivity and excitability in cortical circuitry. The PWK mouse strain, then, represents an opportunity to identify and harness adaptive mechanisms to lengthen cognitive resistance to the normal process of aging.

The high-resolution spine reconstructions performed here and viral-based strategies allowed for specific populations of projection neurons to be compared across mice¹⁶⁶. Past work has relied on disparate techniques, including strategies to label random

neurons with dye-filling methods or counting random synapses by EM, to build a coherent picture of synaptic vulnerability of the aging brain^{52,163,176,290}. By taking advantage of multisite viral strategies to gain projection neuron specificity within the same aging subjects, our results are the beginning of identifying the neural circuits that are vulnerable to age-related disconnection in terms of information flow. Interplay between CA1 of the hippocampus, PFC and RE is thought to be critical for spatial working memory tasks, a cognitive domain affected in aged individuals^{297,317,323}. Since our most striking finding is the strain-dependent loss on aging PFC-to-RE neurons, our results suggest the PFC may be the critical locus of aging in terms of synapse loss. Reductions in PFC-to-RE spine densities in B6 mice was most dramatic from young to middle-aged, which is consistent with previous findings of synapses on PFC neurons from rats^{163,291}, as well as with behavioral findings that animals and humans start to become impaired in working memory function tasks as early as middle-age^{292,317,324,325}. Although CA1 projection neurons appear to maintain their numbers during aging, our results do not imply that CA1 function is intact during aging. Other processes, including activity-dependent spine plasticity at CA1 synapses, might also contribute to age-related deficits in hippocampal-dependent learning or memory function.

At the level of individual synapses, spine structure is a strong determinant of function^{92,155,159}. Specifically, spine head diameter is a correlate of synapse strength, and spine neck of the level of compartmentalization between the synapse and parent dendrite. Past work has focused on age-related modification of head diameter with little evidence for modulation of spine neck morphology³¹⁴. In contrast, our data suggest that both spine head diameter and neck length are readily modified during aging in a way that depends on genetic context. In B6 mice, CA1-to-PFC neurons exhibited very few morphological spine changes with age, consistent with a remarkable population stability during aging. Conversely, PWK mice showed consistent evidence for morphological

spine remodeling on CA1-to-PFC and PFC-to-RE neurons that are suggestive of an overall increase in efficacy between the spine synapse and parent dendrite (**Table 3.1-3.2**). These data are compelling because both spine head and spine neck changes move in unison towards greater synapse efficiency, and their synergism might potentially drive large changes in how excitatory postsynaptic potentials from multiple synapses are integrated in the dendrites.

Recent work from our group has found that PWK mice are resilient to AD-related synaptic changes in CA1-to-PFC neurons during early exponential phases of pathology spread²⁹⁵. As biological age is the primary risk factor for developing neurodegenerative disorders including AD^{11,13}, the data presented here suggest that the PWK mouse strain can be both a model of resilience to cognitive and synaptic changes seen in AD^{53,295}, and resistance to cortical synapse loss seen during normal brain aging. Since the molecular and cellular mechanisms that differentiate successful from unsuccessful agers are still unknown⁷², the findings here could drive the identification of cell-specific processes that maintain synapses throughout aging. Future work should seek to advance this goal by testing additional PFC projection neurons to understand the circuit specificity of resistance, by molecular strategies to uncover the gene(s) required for this resistance, and by using functional studies that interrogate how behavior relates to patterns of neuronal activity in these circuits^{316,326}. Ultimately, such work would set the stage for developing precise therapeutic strategies to promote healthy cognitive aging and resilience to AD across our genetically diverse and aging human population.

Table 3.1 Summary of observed age-related spine responses and predicted consequences to neuronal function in CA1-to-PFC projection neurons.

	Age Groups	Experimental measures			Predicted measures	
		Density	Head Diameter	Neck Length	EPSP spine	EPSP dendrite
C57BL/6J Proximal	Young → Middle-Aged	--	--	↑	--	↓
	Middle-Aged → Aged	--	--	↓	--	↑
	Young → Aged	--	↑	--	↑	--
C57BL/6J Distal	Young → Middle-Aged	--	↓	--	↓	--
	Middle-Aged → Aged	--	--	--	--	--
	Young → Aged	--	--	↓	--	↑
PWK/PhJ Proximal	Young → Middle-Aged	--	↓	↑	↓	↓
	Middle-Aged → Aged	--	↑	↓	↑	↑
	Young → Aged	--	↓	↓	↓	↑
PWK/PhJ Distal	Young → Middle-Aged	--	↓	--	↓	--
	Middle-Aged → Aged	--	↑	↓	↑	↑
	Young → Aged	--	↑	↓	↑	↑

Table 3.2: Summary of observed age-related spine responses and predicted consequences to neuronal function in PFC-to-RE projection neurons.

	Age Groups	Experimental measures			Predicted measures	
		Density	Head Diameter	Neck Length	EPSP spine	EPSP dendrite
C57BL/6J Proximal	Young → Middle-Aged	↓	↑	↓	↑	↑
	Middle-Aged → Aged	--	--	↑	--	↓
	Young → Aged	↓	↑	↑	↑	↓
C57BL/6J Distal	Young → Middle-Aged	↓	↑	--	↑	--
	Middle-Aged → Aged	--	↓	↑	↓	↓
	Young → Aged	↓	--	↑	--	↓
PWK/PhJ Proximal	Young → Middle-Aged	--	--	--	--	--
	Middle-Aged → Aged	--	↑	↓	↑	↑
	Young → Aged	--	↑	↓	↑	↑
PWK/PhJ Distal	Young → Middle-Aged	--	↓	--	↓	--
	Middle-Aged → Aged	--	↑	↓	↑	↑
	Young → Aged	--	--	↓	--	↑

3.4 Methods

3.4.1 Ethics Statement

All research was approved by the Institutional Animal Care and Use Committee (IACUC) at The Jackson Laboratory (approval number 12005 and 20006). Animals were humanely euthanized with 4% tribromoethanol (800 mg/kg). Authors performed their work following guidelines established by “The Eight Edition of the Guide for the Care and

Use of Laboratory Animals” and euthanasia using methods approved by the American Veterinary Medical Association.

3.4.2 Animal husbandry

All mice were bred and housed in a 12/12 hour light/dark cycle on aspen bedding and fed a standard 6% Purina 5K52 Chow diet. Experiments were performed using two mouse strains: C57BL/6J (B6, JAX stock #000664) and PWK/PhJ (PWK, JAX stock #003715). Mice were group housed for entirety of experiments. Experimental cohorts were generated through intercrossing B6 or PWK mice to produce 3-8 female mice per age group. Full mouse information is reported in **Table 3.3**.

Table 3.3: Meta-data for mice and assigned age-groups in Chapter 3.

MouseID	Strain	Sex	DOB	Harvest Date	Age (m)	Age Group
59944	PWK/PhJ	F	11/24/2019	5/21/2022	30	Aged
59246	PWK/PhJ	F	4/24/2020	5/21/2022	25	Aged
59247	PWK/PhJ	F	4/24/2020	5/21/2022	25	Aged
76266	PWK/PhJ	F	3/9/2021	8/8/2022	17	Middle-Aged
76267	PWK/PhJ	F	3/9/2021	8/8/2022	17	Middle-Aged
60938	PWK/PhJ	F	3/16/2021	6/13/2022	15	Middle-Aged
60939	PWK/PhJ	F	3/16/2021	6/13/2022	15	Middle-Aged
60942	PWK/PhJ	F	3/16/2021	6/13/2022	15	Middle-Aged
56621	PWK/PhJ	F	6/23/2021	6/30/2022	12	Middle-Aged
56622	PWK/PhJ	F	6/23/2021	6/30/2022	12	Middle-Aged
56833	PWK/PhJ	F	7/14/2021	6/30/2022	11	Middle-Aged
70328	PWK/PhJ	F	11/5/2021	6/13/2022	7	Young
70329	PWK/PhJ	F	11/5/2021	6/13/2022	7	Young
27811R	PWK/PhJ	F	1/11/2022	6/13/2022	5	Young
2781B	PWK/PhJ	F	1/11/2022	6/13/2022	5	Young
27812L	PWK/PhJ	F	1/11/2022	6/13/2022	5	Young
2430A	C57BL/6J	F	8/9/2022	12/13/2022	4	Young
2430B	C57BL/6J	F	8/9/2022	12/13/2022	4	Young
A0003	C57BL/6J	F	5/31/2022	11/4/2022	6	Young
A0004	C57BL/6J	F	5/31/2022	11/4/2022	6	Young
24311R	C57BL/6J	F	5/10/2022	12/18/2022	7	Young
2432A	C57BL/6J	F	5/10/2022	12/18/2022	7	Young
5551R	C57BL/6J	F	11/23/2021	12/18/2022	13	Middle-Aged
555B	C57BL/6J	F	11/23/2021	12/13/2022	13	Middle-Aged
15721R	C57BL/6J	F	7/13/2021	12/13/2022	17	Middle-Aged
15722R	C57BL/6J	F	7/13/2021	12/18/2022	17	Middle-Aged
60840	C57BL/6J	F	2/16/2021	12/13/2022	22	Aged
A3924	C57BL/6J	F	12/2/2020	11/4/2022	23	Aged
A3926	C57BL/6J	F	12/2/2020	12/18/2022	24	Aged

3.4.3 Intracranial viral injections

Recombinant adenoassociated viral (AAV) vectors were used to drive Cre-recombinase (AAVretro-Cre)¹⁶⁶, Cre-dependent EGFP (serotype 2/1, AAV-FLEX-*rev*-EGFP)^{166,167}, Flp-recombinase (AAVretro-CAG-FLPo), and FLP-dependent tdTomato (serotype 2/1 AAV-CAG-FRT-*rev*-TdTomato)³²⁷. AAVretro-CAG-FLPo and AAV-CAG-FRT-*rev*-TdTomato were gifts from Janelia Viral Tools (Addgene plasmid #183412, <http://n2t.net/addgene:183412>, RRID:Addgene_183412; Addgene plasmid #191203, <http://n2t.net/addgene:191203>, RRID:Addgene_191203). The titers of each virus were as follows (in genomic copies/mL): AAVretro-Cre, 1×10^{12} , AAV-FLEX-*rev*-GFP, 1×10^{13} , AAVretro-CAG-FLPo, 1×10^{13} ; AAV-CAG-FRT-*rev*-TdTomato, 1×10^{13} . A 1:1 ratio of AAVretro-CAG-FLPo and AAV-FLEX-*rev*-GFP was mixed and 45 nL injected into ventral prefrontal cortex (PFC) over 5 minutes; 45-50 nL (per each D/V coordinate) of AAV-CAG-FRT-*rev*-TdTomato was injected in CA1 (CA1) over 10 minutes; AAVretro-Cre was diluted 1:10 with sterile saline and 45 nL injected into nucleus reunions (RE) over 5 minutes. Since PWK brain volumes are smaller than B6, injection coordinates were adjusted based on pilot experiments to determine injection sites. The coordinates for each injection were as follows (in mm: posterior relative to bregma, lateral relative to midline, and ventral relative to pial surface): B6 PFC (+1.75, -0.95, and -2.6), B6 CA1 (-3.5, -3.4, and -2.7/-2.5/2.0), B6 RE(-1.1, -1.2, -4.15); PWK PFC (+1.45, -0.9, and -2.3), PWK CA1 (-3.5, -3.3, and -2.75/-2.5/-2.0), PWK RE(-1.0, -1.0, -3.2). RE and PFC injections were performed with the mouse tilted at a 15° angle. At each site the injection pipette was left in place for 3-5 minutes then slowly retracted at a rate of 10 μ m/s from the brain. After surgery mice were singly housed, monitored for 5 days, and euthanized approximately 3-4 weeks post injection.

3.4.4 Tissue harvest and brain sectioning

Mice were euthanized with an intraperitoneal lethal dose of Tribromoethanol (800 mg/kg), followed by transcardial perfusion with 45 mL ice-cold 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered saline, in accordance with IACUC protocols (12005 and 20008). Brains were removed and placed in 5mL ice cold 4% PFA at 4°C for 24 hours, then placed into storage buffer (1XPBS + 0.1% Sodium Azide) for long-term storage at 4°C. Brains were coronally sectioned at 50µm thickness and placed in storage buffer at 4°C until needed for imaging. Approximately 6 frontal PFC sections containing EGFP+ dendrites, and 6 sections with distal CA1 TdTomato+ dendrites were mounted on slides and coverslipped using Vectashield Hardset mounting media (Vector Laboratories #H140010).

3.4.5 Dendritic spine imaging and analysis

Slices were imaged on an SP8 confocal microscope equipped with a 63X objective (oil immersion), images collected at 50 nm pixel sizes with 0.1 µm z-steps, and stacks deconvolved using Leica Lightning software. 10 dendrites per compartment (e.g. basal, apical oblique, tuft) were captured per mouse. Each image was exported as TIFF format (in ImageJ version 2.9.0/1.53t) and imported into NeuronStudio³²⁸ for analysis of dendritic spine densities and morphologies. Since the effectiveness of a synaptic input on the dendritic tree is influenced by the diameter of the parent dendrite³²⁹, we ensured all dendritic calibers incorporated into this analysis were thin (i.e., under 1 µm in diameter) (**Figure 3.3A, 3.5A, 3.7A, 3.9A**). Density measurements were acquired by first reconstructing the dendritic cable followed by semi-automated spine identification. Cumulative distributions of assigned spine head diameters (HEAD.DIAMETER) and neck lengths (MAX.DTS) were analyzed by Kolmogorov-Smirnov tests, and through a

quartile-based analysis. In this latter analysis, spines within dendritic compartments from each strain were pooled across treatment groups to create a population, and the first and last quartiles determined. From each branch, spines belonging to the first quartile (Q1, smallest) and last quartile (Q4, largest) were identified and the density for each quartile per branch was calculated. Data were analyzed with each dendrite representing an individual data point.

3.4.6 Statistical analysis

Data were analyzed blinded to strain and age group. All statistical analyses were performed in GraphPad Prism software (v9.5.1) except for Kolmogorov-Smirnov tests which were performed using R (v4.2.2). Data from B6 and PWK mouse strains were analyzed separately. To assess age effects within each strain, one-way ANOVAs were computed followed by Bonferroni post hoc tests. Within-group differences between Q1 and Q4 densities for spine head diameter and neck length were determined using nonparametric two-tailed t-tests. Bonferroni corrections for multiple comparisons were performed on nominal p-values from resultant K-S tests.

3.4.7 Data and resource sharing plan

All mouse strains are available through The Jackson Laboratory. All reagents in this study are commercially available. Raw data (DOI: 10.6084/m9.figshare.23620752) and images from the figures (DOI: 10.6084/m9.figshare.23620755) are available via Figshare.

3.5 Acknowledgements

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3.6 Author Contributions

S.E.H., E.B.B. and G.R.H. designed the study. S.E.H. generated and maintained mouse cohorts, and S.E.H. and E.B.B. performed the intracranial AAV injections. S.E.H. euthanized mice and collected tissues. S.E.H. and E.W.N. sectioned brains and prepared slides for imaging. S.E.H. and E.W.N. imaged slices and analyzed final data. E.B.B. provided training in intracranial injections, confocal microscopy imaging of dendrites and dendritic spines, and NeuronStudio analysis. E.B.B. and G.R.H. advised on all data analysis, data interpretation and manuscript preparation. S.E.H., E.W.N., E.B.B. and G.R.H. wrote and edited the manuscript. All authors approved the final version.

Chapter 4: Control of hippocampal synaptic plasticity by microglia-dendrite interactions depends on genetic context in mouse models of Alzheimer's disease³

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4.1 Introduction

Individuals affected by Alzheimer's disease (AD) exhibit decline across cognitive domains including learning and memory, and executive functioning⁹. The accumulation of amyloid beta ($A\beta$) plaques and neurofibrillary tangles that define AD are often associated with progressive yet selective patterns of synaptic disruption that emerge in entorhinal and hippocampal circuits before becoming widespread across frontal and parietal association areas^{72,178}. The selective vulnerability of these circuits occurs alongside robust neuroinflammation¹²⁷, and one hypothesis posits that activation of microglia in response to $A\beta$ plaque deposition is a causal event driving neural circuit disruption¹⁹⁴. This is supported by the known role of microglia regulating synaptic connectivity during development and in the healthy, adult brain^{129,193}, and observations that microglia depletion in AD mouse models reduces late-stage hippocampal synapse loss^{116,147}. However, these experiments are confounded by the coincident observation that complete microglia depletion caused spatial redistribution of parenchymal $A\beta$ plaques to the cerebrovasculature^{139,330}. Thus, the precise relationship between $A\beta$ deposition, microglia activation, and neuronal synapse disruption in specific AD-vulnerable circuits is still to be determined.

Age at symptom onset is variable among AD patients carrying similar rare high-risk mutations in *APP* and *PSEN1* and bearing similar plaque loads, implicating genetic context as an important factor controlling AD progression²². Cognitive resilience has also been observed in subsets of late onset AD (LOAD) patients, suggesting additional genetic components shape the cellular events mediating cognitive decline^{331,332}. However, previous studies of synaptic dysfunction using AD mouse models have been performed almost exclusively on the inbred C57BL/6J (B6) genetic background. Incorporation of genetic diversity into AD mouse models has become better appreciated

through studies of traditional transgenic AD mouse models on genetically diverse mouse strains^{45,48,53,54,333}. We have shown that despite identical patterns of A β plaque deposition, wild-derived PWK/PhJ (PWK) mice carrying the *APP/PS1* transgene (PWK.*APP/PS1*) exhibit cognitive resilience compared to traditionally-studied B6.*APP/PS1* inbred mice⁵³. Intriguingly, PWK and PWK.*APP/PS1* mice also contain different proportions of transcriptionally-defined microglia states compared to B6, suggesting microglia may be a potential factor mediating resilience to brain A β deposition⁵⁴.

Here, we examined how genetic context influences the role of microglia on synaptic changes during A β plaque deposition. We used a viral approach¹⁶⁶ to gain genetic access to an AD-vulnerable neuronal circuit³³⁴ connecting hippocampal area CA1 to the prefrontal cortex (PFC) in B6 and PWK wild-type (WT) and *APP/PS1* transgenic (TG) mice, permitting rigorous comparisons across equivalent neuronal populations. The CSF1R inhibitor PLX5622 was used to deplete brain microglia while keeping A β plaque deposition unaltered. Dendritic spine density (a proxy for synaptic number) and spine morphology (a proxy for synaptic stability, plasticity and strength^{92,155,335}) was quantified across the dendritic compartments of CA1 pyramidal cells. We found a microglia-dependent increase in spine density on proximal oblique dendrites from B6.*APP/PS1* mice that was accompanied by a shift towards smaller spines; both effects were completely absent in PWK.*APP/PS1* mice. Further supporting a context-dependent role for microglia in synapse plasticity during AD, B6.*APP/PS1* but not PWK.*APP/PS1* mice showed differential remodeling of synapses on individual branches that were directly contacted by microglia processes. Collectively, these results provide strong evidence that the mechanisms driving synaptic responses to amyloid depend on genetic context.

4.2 Methods

4.2.1 Ethics Statement

All research was approved by the Institutional Animal Care and Use Committee (IACUC) at The Jackson Laboratory (approval number 12005 and 20006). Animals were humanely euthanized with 4% tribromoethanol (800mg/kg). Authors performed their work following guidelines established by “The Eight Edition of the Guide for the Care and Use of Laboratory Animals” and euthanasia using methods approved by the American Veterinary Medical Association.

4.2.2 Animal husbandry

All mice were bred and housed in a 12/12 hour light/dark cycle on aspen bedding and fed a standard 6% Purina 5K52 Chow diet unless otherwise stated. Pilot experiments were performed on two mouse strains: C57BL/6J (B6, JAX stock #000664) and PWK/PhJ (PWK, JAX stock #003715). Experimental cohorts were generated to produce 3 female mice per group. Mice were group housed for entirety of experiments. Primary experiments were performed on two additional mouse strains: B6.Cg-Tg(APP^{swe}, PSEN1^{dE9})85Dbo/Mmjax (JAX stock #005864) and PWK.*APP/PS1* (JAX stock #25971). Experimental cohorts were generated to produce 6 female mice per group (12 *APP/PS1* carriers and littermate wild-type controls). However, due to increased seizure-induced mortality of B6.*APP/PS1* mice³³⁶, final cohorts for this strain were n=6 for TG control diet and n=5 for TG PLX5622 diet. Mice were initially group housed until 2.5 months of age (2 weeks before intracranial injections) mice were singly housed to avoid fighting-induced mortality among PWK.*APP/PS1* mice.

4.2.3 Intracranial viral injections

Recombinant adenoassociated viral (AAV) vectors were used to drive Cre-recombinase (AAVretro-Cre)¹⁶⁶, and Cre-dependent EGFP (serotype 2/1, AAV-flex-*rev*-EGFP)¹⁶⁷. The titers of each virus were as follows (in genomic copies/mL): AAVretro-Cre, 1×10^{12} ; AAV-flex-*rev*-GFP, 1×10^{13} . 30nL of AAVretro-Cre was injected into ventral prefrontal cortex (PFC) over 5 minutes, and 45-50nL (per each D/V coordinate) of AAV-flex-*rev*-GFP was injected in CA1 (CA1) over 10 minutes. Since PWK brain volumes are smaller than B6, injection coordinates were adjusted based on pilot experiments to determine injection sites. The coordinates for each injection were as follows (in mm: posterior relative to bregma, lateral relative to midline, and ventral relative to pial surface): B6 PFC (+1.75, -0.95, and -2.6), B6 CA1 (-3.5, -3.4, and -2.7/-2.5/2.0); PWK PFC (+1.45, -0.9, and -2.3), PWK CA1 (-3.5, -3.3, and -2.75/-2.5/-2.0). At each site the injection pipette was left in place for 3-5 minutes then slowly retracted at a rate of $10 \mu\text{m/s}$ from the brain. After surgery mice were singly housed until sacrifice at 8 months of age.

4.2.4 Microglia depletion with PLX5622

PLX5622 was acquired from Chemgood (#C1521) and formulated in Purina 5K52 mouse chow diet at a concentration of 1200mg/kg (ppm) by Research Diets Inc, followed by 10-20 kGy gamma irradiation. Chemical purity and proper diet concentration were validated through HPLC and mass spectrometry analysis through Chemgood and JAX metabolomics core (detected 1030mg/kg purified from 1 pellet of diet). Mice were placed on diet at 4 months of age (4m) and left of diet until 8 months of age (8m). Mice were monitored weekly for food consumption and weighed monthly.

4.2.5 Tissue harvest and brain sectioning

Mice were euthanized with an intraperitoneal lethal dose of Tribromoethanol (800mg/kg), followed by transcardial perfusion with 45mL ice-cold 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered saline, in accordance with IACUC protocols (12005 and 20008). Brains were removed and placed in 5mL ice cold 4% PFA at 4°C for 24 hours, then placed into storage buffer (1XPBS + 0.1% Sodium Azide) for long-term storage at 4°C. Brains were sectioned at alternating thicknesses of 200µm and 50µm and kept in storage buffer at 4°C until needed for imaging.

4.2.6 Immunofluorescence analyses of microglia and amyloid-β plaques

50µm sections with EGFP+ dendrites were permeabilized with 1XPBS + 1% TritonX-100 (PBT), blocked for 12 hours at 4°C in PBT + 10% normal donkey/goat serum, washed once with PBT, and incubated in primary antibody solution containing rabbit anti-IBA1 (1:300, Wako #01919741) or chicken anti-IBA1 (1:500, Synaptic Systems #234009). After primary incubation for 72 hours at 4°C, sections were washed 3 times with PBT and incubated in secondary antibodies (goat anti-rabbit Alexa Fluor 568 (1:500 in PBT, ThermoFisher #A11011) or donkey anti-chicken Alexa Fluor 647 (1:500 in PBT, Jackson ImmunoResearch #703605155)) for 24 hours at 4°C. Sections were washed with PBT, counterstained with DAPI (ThermoFisher #D3571, 0.2mg/mL diluted 1:1000 in PBS), washed with PBS, and mounted with Vectashield Hardset (Vector Laboratories #H140010) mounting media. For assessing amyloid-β plaque pathology, additional 50µm sections underwent similar protocols, with X34 steps occurring before primary antibodies were applied. X34 solution was prepared by diluting 0.4mg X34 (Sigma #SMIL1954) in 4mL 200 proof ethanol, and 6 mL distilled water (diH₂O). Sections were incubated in X34 solution for 10 minutes, rinsed in diH₂O for 3 minutes, incubated in 0.02M sodium

hydroxide (NaOH) for 5 minutes, and washed in PBS. X34 staining was then proceeded by primary antibody staining. Additionally, since X34 and DAPI fluorescence are in overlapping channels, sections that underwent X34 staining were counterstained with TOPRO3 (Invitrogen #13605, 1:1000 diluted in PBS) instead of DAPI.

Images were captured using two methodologies. EGFP+ sections that were stained with anti-IBA1 and DAPI were imaged on a Leica SP8 confocal microscope at 40X magnification, with each tile captured at 512 x 512 pixel frames using 2 μ m z-stack sizes. Sections that were stained for X34, anti-IBA1 and TOPRO3 were imaged on a Leica Versa slide scanner at 10X magnification, capturing and merging individual tiles. Analysis was performed using ImageJ2 (version 2.9.0/1.53t), where regions of interest (ROI) were outlined for SLM, SR, and SO as structurally visualized by the DAPI/TOPRO3 counterstains. For IBA1+DAPI+ quantification, maximum projections were generated from stacks, individual channels isolated, and default thresholds applied for IBA1 and DAPI to create binary images. IBA1 and DAPI binary images were merged for overlapping signal, followed by quantification of spots using the particle analyzer function (size threshold of 5-infinity pixels). For X34 quantification, the X34 channel was isolated from each image, thresholded to create a binary image, and quantified for total X34+ area and plaque number using the particle analyzer function. For quantification of plaque associated (PAM) and non-plaque associated (NPAM) microglia, 100 μ m in diameter ROIs were outlined around areas with X34+ plaques in SLM of each *APP/PS1* mouse. In the thresholded IBA1 channel from each imaged brain, IBA1+ area was quantified for each PAM ROI using the particle analyzer function. The sum of total IBA1+ area from each measured SLM was obtained and subtracted from the total quantified IBA1+ area to obtain NPAM IBA1+ area. When multiple SLM were analyzed per mouse, mean values from each PAM and NPAM ROI were calculated to obtain individual mouse

statistics. Each quantified measure was normalized to area of ROI to obtain accurate densities.

4.2.7 Cerebral Amyloid Angiopathy (CAA) Scoring

CAA severity was semi-quantitatively evaluated as described previously³³⁷. Images of X34+ plaques from transgenic *APP/PS1* mice were evaluated for CAA by three individual scorers, each blinded to the strain and treatment. Each image was assigned a semi-quantitative score ranging from 0 to 4 by the criteria as follows: 0 = no amyloid in vessels, 0.5 = scattered amyloid observed in leptomeninges, 1 = scattered amyloid in leptomeningeal and cortical vessels, 2 = strong circumferential amyloid deposition in multiple cortical and leptomeningeal vessels, 3 = widespread strong amyloid deposition in leptomeningeal and cortical vessels, and 4 = extravasation of amyloid deposition accompanied by dysphoric amyloid. For each image, the mode of the three scorers was obtained. If multiple images were acquired for each mouse, the mean CAA score was calculated to obtain a representative mouse score.

4.2.8 Dendrite imaging, reconstruction, and analysis

200 μ m sections containing EGFP+ dendrites were identified using a fluorescence dissecting light microscope, were mounted on slides with 2 stacked 120 μ m imaging spacers (Electron Microscopy Sciences #7032720S) in Vectashield mounting media (Vector Laboratories #H100010). Images were acquired on a Leica SP8 confocal microscope at 40X magnification (oil immersion), 512 x 512 pixel dimensions, 1.25X digital zoom, 2 μ m z-steps, and depth-dependent detector gain compensation to maintain signal strength through the depth of the stack. Images were converted into TIFFs, followed by dendritic reconstructions in NeuronStudio³²⁸ (v0.9.92) software. For dendrite

reconstructions, a maximum of 5 dendritic arbors from each compartment (e.g. basal, apical oblique, tuft) per mouse were reconstructed. Dendritic compartments were defined as follows: basal dendritic compartments included multiple origins, each of which emanate from the soma and traverse away from the SR; oblique dendritic compartments originate as a thick primary branch from the soma and gives rise to multiple terminal oblique dendrites; and tuft dendritic compartments originate at the site of the primary apical dendrite bifurcation as the dendrite enters the SLM and continues at individual branch termination. After reconstructions, three dimensional Sholl analysis was performed on each dendrite using concentric circles spaced 20 μ m apart, originating at the soma for basal and oblique, and at the primary bifurcation for tuft dendrites. Summary statistics including total dendritic length and number of branch points were also acquired from this analysis, and each reconstructed neuron was reported as an individual measure.

4.2.9 Dendritic spine imaging and analysis

EGFP+ dendrites were imaged on an SP8 confocal microscope equipped with a 63X objective (oil immersion), images collected at 50nm pixel sizes with 0.1 μ m z-steps, and stacks deconvolved using Leica Lightning software. 5-15 dendrites per compartment (e.g. basal, apical oblique, tuft) were captured per mouse. Slices that underwent co-labeling with anti-IBA1 antibodies had an additional channel captured for the secondary antibody signals. Each image was exported as TIFF format and imported into NeuronStudio³²⁸ for analysis of dendritic spine densities and morphologies. Density measurements were acquired by first reconstructing the dendritic cable followed by semi-automated spine identification. Cumulative distributions of assigned spine head diameters were analyzed by Kolmogorov-Smirnov tests, and through a quartile-based

analysis. In this latter analysis, spines within dendritic compartments from each strain were pooled across treatment groups to create a population, and the first and last quartiles determined. From each branch, spines belonging to the first quartile (Q1, smallest) and last quartile (Q4, largest) were identified and density for each quartile per branch was calculated. Data were analyzed with each dendrite representing an individual data point.

4.2.10 Microglia touch: proximal versus distal analysis

Images from 50 μ m sections that were co-labeled with anti-IBA1 were assessed for microglia-dendrite interactions by merging the EGFP (488) and IBA1 (568/647) channels in each slice in the z-stack. Images were then classified as Touch+ or Touch- based on whether the dendritic signal was physically overlapping with an IBA1+ process. Within the group of images that were Touch+, the exact x-y-z coordinates of the point of interaction were identified, and spine densities/morphologies calculated for the region of the dendrite 5 μ m on either side of the location of the touch (proximal, 10 μ m dendritic segment total). Spine densities and morphologies were gathered for the dendritic region that was 10 μ m adjacent to the dendritic region proximal to the microglia contact (distal). If the microglia contact appeared in the center of the dendrite, two distal 10 μ m dendritic regions were created on either side of the proximal zone, and mean spine density/morphology from distal zones calculated so that each proximal dendrite included a paired corresponding distal dendrite. Spine densities were calculated by each reconstructed dendrite, and spine sizes analyzed by each measured spine.

4.2.11 Pilot: tissue harvest and brain sectioning

Mice were euthanized as described previously, followed by cardiac puncture and transcardial perfusion with 1XPBS in accordance with IACUC protocols. Blood collected from cardiac puncture was placed in EDTA-coated microtubes (BD Biosciences #363706) at room temperature until processing for flow cytometric analysis. Brains were removed and hemisected. Left brain hemispheres were placed in ice cold homogenization solution (Hank's balanced salt solution [HBSS] with 15mM HEPES and 0.5% glucose), and immediately processed for flow cytometric analysis. Right brain hemispheres were placed in 5mL ice cold 4% paraformaldehyde (PFA) at 4°C for 24 hours, 10% sucrose for 24 hours, 30% sucrose for 24 hours, frozen and stored at -80°C for long-term storage.

4.2.12 Pilot: brain homogenization, myeloid cell preparation and FACs analysis

Brains were homogenized and myeloid cells isolated as described previously⁵⁴. All hemispheres were homogenized on ice and performed using ice cold solutions to avoid myeloid cell activation. Each hemisphere was minced using a scalpel, followed by homogenization with a 15mL PTFE tissue grinder (4-5 strokes) in 2mL homogenization buffer. The suspension was transferred to a 50mL tube and passed through a pre-wet (with homogenization buffer) 70µm strainer. The suspension was transferred to a 15mL tube and spun in a centrifuge at 500g for 5 minutes at 4°C. After discarding the supernatant the cell pellet was resuspended in 2mL MACS buffer (PBS + 5% bovine serum albumin (BSA) + 2mM Ultrapure EDTA) for myelin removal procedure. 200µL Myelin Removal Beads (Miltenyi Biotec #130096433) was added to the cell suspension and mixed by gently pipetting. The cell suspension was divided equally into two 2mL microcentrifuge tubes and incubated at 4°C for 10 minutes. After incubation 1mL MACS

buffer was added to each tube and centrifuged for 30s at 9300g at 4°C. After discarding supernatant, cell pellets were resuspended in 1.5mL MACS buffer per tube, and transferred to two pre-wet LD columns (Miltenyi Biotec #130042901). The flow through was collected in 50mL tubes on ice, and LD columns rinsed twice with 2mL MACS buffer. The final flow-through with washes were divided into multiple 2mL centrifuge tubes and centrifuged at 9300g for 30s at 4°C. After discarding supernatants, cell pellets were resuspended in 1mL 1XPBS. Since additional debris was still present, samples underwent debris removal protocol. After resuspension with 1XPBS, samples were transferred to 15mL conical tubes and 900µL Debris Removal solution (Miltenyi Biotec #130109398) was added to each tube. Each mixture was carefully and slowly overlaid with 4mL 1XPBS, and centrifuged at 3000g for 4 minutes at 4°C. The top 2 interfaces were removed from the density gradient formed after centrifugation, and the bottom layer was saved on ice. 10mL 1XPBS was added to each tube, inverted gently, and centrifuged at 1000g for 10 minutes at 4°C. Supernatants were removed, and pellets resuspended in 1mL 1XPBS and stored on ice. Flow cytometric analysis, each sample was stained with DAPI, CD45 BV605 (clone 30-F11, BD Biosciences #563053, 1:240), and CD11b PE (clone M1/70, Biolegend #101207, 1:960), followed by processing on a FACSymphony A5 cytometer, and analysis using FlowJo (v10) software.

4.2.13 Pilot: Blood preparation and FACs analysis

Blood collected from cardiac punctures were first processed by lysing red blood cells, followed by staining with the following antibodies: CD11c FITC (clone N418, TONBO #350114U100, 1:600), CD19 PerCP-Cy5.5 (clone 1D3, TONBO #650193U100, 1:480), CD11b PE (clone M1/70, Biolegend #101207, 1:960), CD3e PE-CD594 (clone 145-2C11, BD Biosciences #562286, 1:120), CD62L PE-Cy7 (clone MEL-14, TONBO

#600621U100, 1:600), CD4 APC (clone RM4-5, Biolegend #100516, 1:480), CD8a A700 (clone 53-6.7, Biolegend #100729, 1:600), Ly6G BV421 (clone 1A8, BD Biosciences #562737, 1:480), CD45 BV605 (clone 30-F11, BD Biosciences #563053, 1:240), B220 BUV496 (clone RA3-6B2, BD Biosciences #564662, 1:120). Samples were processed on a FACSymphony A5 cytometer and analyzed using FlowJo (v10) software.

4.2.14 Statistical analysis

Data were analyzed blinded to genotype and treatment group. All statistical analyses were performed in GraphPad Prism software (v9.5.1) except for Kolmogorov-Smirnov tests which were performed using R (v4.2.2). Data from B6 and PWK mouse strains were analyzed separately. To assess treatment and genotype effects within each strain, two-way ANOVAs were computed followed by Bonferroni post hoc tests. Differences between treatment groups from plaque associated and non-plaque associated microglia area were assessed using nonparametric two-tailed t-tests within each strain. Comparisons of spine densities from this study to previously published array tomography and electron microscopy findings were tested using one-way ANOVA followed by Bonferroni post hoc tests. Quartile-based analyses to tests for differences in Q1 and Q4 densities between quartiles across groups were performed within strain with one-way ANOVA followed by Bonferroni post-hoc tests. Within-quartile effects on spine density across genotype/treatment groups were assessed with two-way ANOVA within strains followed by Bonferroni post-hoc tests. To determine differences in spine density within strain/treatment/genotype group based on microglia contact (Touch+ vs Touch-), two-tailed nonparametric unpaired t-tests were performed. Within a dendritic segment containing and microglial contact, spine densities between proximal versus distal regions in relation to microglial touch were evaluated using nonparametric two-tailed paired t-tests within strain/treatment/genotype group.

4.2.15 Materials and data availability

All mouse strains are available through The Jackson Laboratory. All reagents in this study are commercially available. Raw data (10.6084/m9.figshare.22713493) and images from the figures (10.6084/m9.figshare.22713685) are available via Figshare.

4.3 Results

4.3.1 CSF1R inhibition depletes microglia without altering plaque pathology in female *APP/PS1* mice

To determine how microglia influence CA1 neurons we formulated the CSF1R inhibitor PLX5622 in mouse diet as described previously¹³⁹. A 3-week pilot study was performed to determine the safety and efficacy of PLX5622 diet in adult (2.5m) female PWK compared to B6 mice. Flow cytometric analysis of isolated myeloid cells from brain hemispheres and immunofluorescence analysis of dorsal CA1 found that PLX5622 diet significantly depleted microglia by 50% in B6, and 80% in PWK (**Figure 4.1A-C**). No within-strain differences in body weights or quantity of diet consumption were observed between PLX5622 and control diet groups. Flow cytometric analysis of peripheral blood revealed no effects on composition of major immune cell populations (**Figure 4.1D-M**).

To test how genetic context controls amyloid- and microglia-dependent impacts on CA1 circuit vulnerability, we generated cohorts of B6.*APP/PS1* and PWK.*APP/PS1* TG female mice and WT littermate counterparts (n=12 per strain/genotype group). At 3 months of age, we performed dual intracranial injections of recombinant adenoassociated virus (AAVretro-Cre in prefrontal cortex (PFC) and FLEX-*rev*-EGFP in CA1, see **Methods**) to drive EGFP expression in CA1 neurons that project to the PFC. At 4 months of age (when *APP/PS1* plaque deposition is first observable⁴¹), we placed 6 mice per strain/genotype group on PLX5622 diet and left 6 mice/group on Purina 5K52

control diet for 4 months until all mice reached 8 months of age (**Figure 4.2A**). Mice were perfused with fixative, coronally sectioned and immunolabeled for markers of amyloid pathology (X34) and microglia (IBA1). The laminar structure in CA1 reflects distinct afferent pathways, so data were analyzed across subregions: stratum lacunosum moleculare (SLM), stratum radiatum (SR), and stratum oriens (SO).

With control diet, the density of microglia was several-fold higher in SLM compared to SR or SO. Both strains, regardless of *APP/PS1* genotype, showed significant PLX5622-mediated reductions of microglia (**Figure 4.2B, Figure 4.1N**). Depletion efficiencies were dependent on laminae, with greater microglia depletion in SO or SR compared to SLM. We examined X34+ A β plaque pathology and found that plaque density varied across CA1 laminae but did not differ between PLX5622 and control diet animals of each strain (**Figure 4.2C, Figure 4.1N**). Unlike previous reports^{139,330}, PLX5622 treatment did not result in increased cerebral amyloid angiopathy (CAA)³³⁷ (**Figure 4.1O**). This could be due to the remaining microglia compacting around plaques, preventing abnormal vascularization and CAA. To test this, we compared plaque-associated microglia (PAM, defined as IBA1+ microglia localized within 100 μ m diameter circle from plaque center) to non-plaque associated microglia (NPAM) in SLM and found that while both PAM and NPAM were significantly depleted, PAM remained in a much higher proportion to NPAM in PLX5622-treated animals (**Figure 4.2D, Figure 4.1P**). This approach allowed for specific evaluation of microglia-neuron interactions without confounding changes to A β plaque pathology.

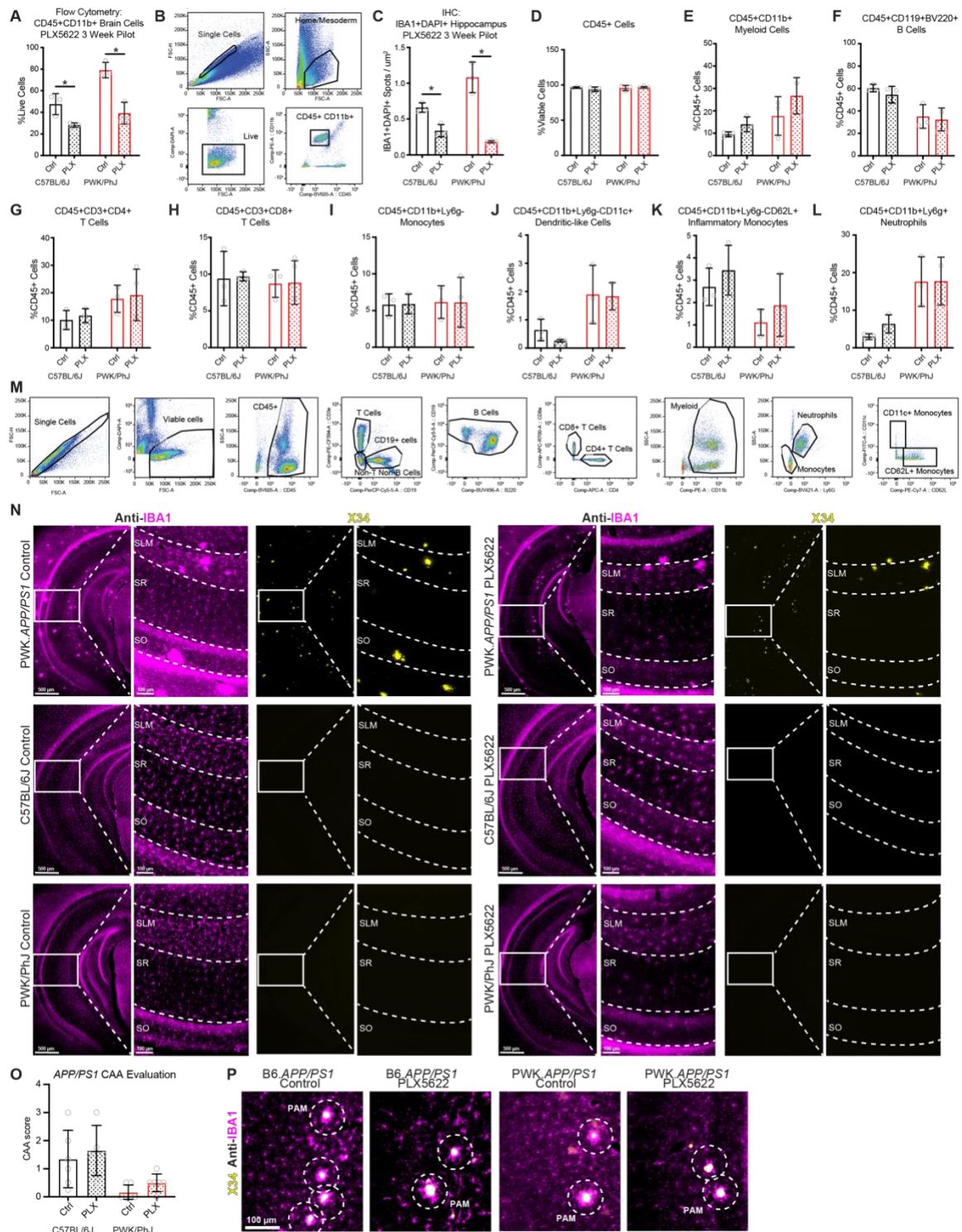


Figure 4.1: Evaluation of PLX5622-mediated microglia depletion across B6 and PWK mice, related to Figure 4.2.

(A-B) Flow cytometric analysis (A) and gating strategy (B) of CD45+CD11b+ cells isolated from brain hemispheres of B6 and PWK mice treated for 3 weeks with Control diet or PLX5622 diet. Data presented as percent (%) of live cells. Two-way ANOVA detected significant (p<0.05) treatment, strain, and interactions.

(C) Immunohistochemical analysis of CA1 anti-IBA1 fluorescence from B6 and PWK mice treated for 3 weeks with Control control or PLX5622 diet. Two-way ANOVA detected significant ($p < 0.05$) treatment and interactions.

(D-M) Flow cytometric analysis of peripheral blood from B6 and PWK female mice treated with Control diet or PLX5622 diet for 3 weeks. Blood cell populations were quantified: CD45+ cells (D), CD45+CD11b+ myeloid cells (E), CD45+CD19+BV220+ B cells (F), CD45+CD3+CD4+ helper T cells (G), CD45+CD3+CD8+ cytotoxic T cells (H), CD45+CD11b+Ly6g- monocytes (I), CD45+CD11v+Ly6g-CD11c+ dendritic-like cells (J), CD45+CD11b+Ly6g-CD62L+ inflammatory monocytes (K), and CD45+CD11b+Ly6g+ neutrophils. CD45+ cells in (D) reported as percent (%) of live cells. Populations quantified in (E-L) reported as % of CD45+ cells. Example gating strategy for peripheral blood analysis is depicted in (M). Two-way ANOVA identified significant ($p < 0.05$) strain effects for (E), (F), (G), (J), (K), and (L).

(N) Images of IBA1+ microglia (magenta) and X34+ A β plaques (yellow) across CA1 sub-regions part of the long-term PLX5622 study. SLM = stratum lacunosum moleculare, SR = stratum radiatum, SO = stratum oriens.

(O) Scoring of CAA severity across *APP/PS1* mice. Nonparametric two-tailed t-tests identified no significant within-strain differences between control Control and PLX5622 treated animals.

(P) Images corresponding to **Figure 4.2D** analysis of plaque-associated microglia (PAM) across *APP/PS1* mice. Images taken at 10X magnification, and regions of interest (100 μ m in diameter) identified as X34+ and X34- across the SLM (5/mouse) in X34 channel. Separately, circular regions overlayed on corresponding IBA1 channels, and IBA1+ area quantified for each region. Depicted images are merged IBA1 (magenta) and X34 (yellow) 10X images, with example circular ROIs outlined for PAM.

All data presented at mean $\pm$ SD, with individual measures plotted as grey points.

Statistical analyses performed on B6 and PWK mouse strains together. For (A), (C)-(L)

*adjusted $p < 0.05$ Bonferroni post-hoc tests.

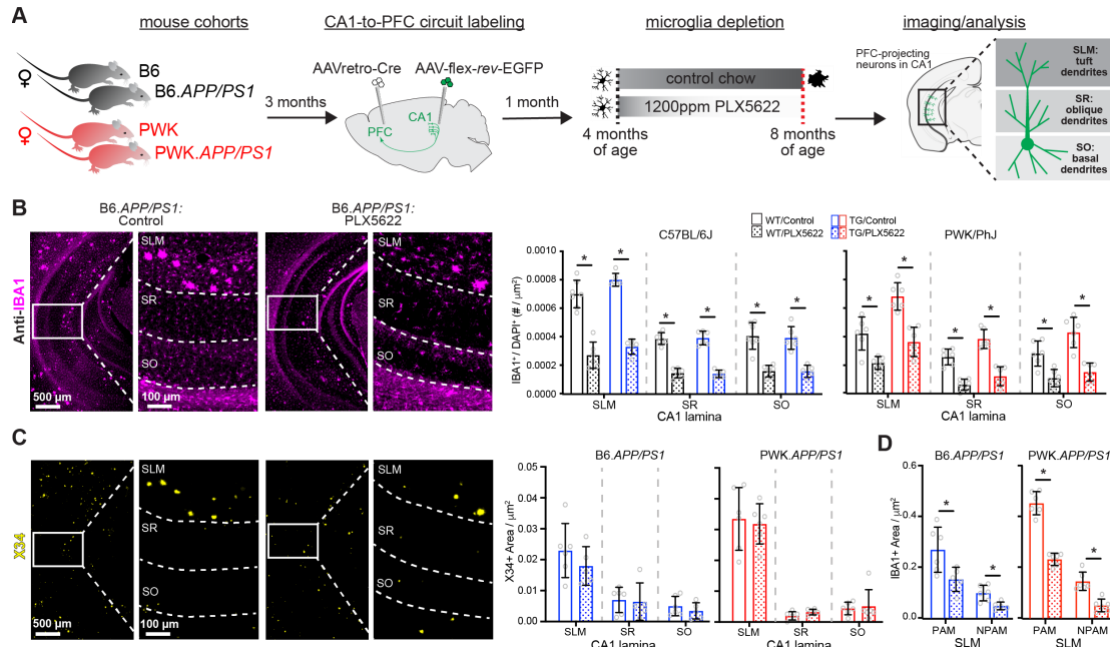


Figure 4.2: Evaluation of microglia composition and amyloid pathology across CA1 in WT and *APP/PS1* mice.

(A) Experimental outline (see **Methods** for additional details). **(B)** IBA1+ microglia from B6.*APP/PS1* TG control and PLX5622 mice (left). Quantification of IBA1+/DAPI+ microglia across CA1 lamina (right). Datapoints represent individual mice ($n=5-6$ /group); error bars are $\pm$ SD; asterisks denote comparisons ($p<0.05$) identified between control and PLX5622 groups (right) after corrections for multiple comparisons. SLM, stratum lacunosum moleculare; SR, stratum radiatum; SO, stratum oriens. **(C)** X34+ A β plaques in B6.*APP/PS1* TG control and PLX5622 mice (left). Quantification of X34+ A β plaque area across CA1 lamina (right), plotted as described above. **(D)** Quantification of IBA1+ area from SLM defined as plaque-associated (PAM) or non-plaque associated microglia (NPAM). Points represent mean values calculated for individual mice and analyzed with two-tailed nonparametric t-tests. Statistical analyses performed on B6 and PWK separately. For (B)-(C) *adjusted $p<0.05$ Bonferroni post-hoc tests. For (D) * $p<0.05$ nonparametric two-tailed t-test.

4.3.2 Strain-specific effects of microglia depletion on proximal dendritic synapses of CA1-to-PFC projection neurons

The vast majority of excitatory synaptic inputs to CA1 pyramidal cells are made onto proximal oblique or basal dendrites^{301,338}. Since these dendritic compartments are close to the site of action potential generation, synapses formed onto these branches strongly affect the output patterns of the cell. Previous work has highlighted structural remodeling

of proximal dendrites in response to A β pathology, an effect that changes the integrative properties of the dendrites³³⁹. We first examined dendritic structure in SR via three-dimensional reconstructions of CA1-to-PFC projection neurons and found no differences among dendritic lengths or branch points across B6 mice regardless of genotype or treatment, but a significant increase in dendrite length between PWK WT and PWK TG PLX5622 mice (**Figure 4.3A**). While Sholl analyses revealed no significant change in dendritic length at specific distances from the soma in B6 mice, both treatment and genotype effects were present in PWK such that lengths were increased in PWK TG PLX5622 compared to PWK WT control mice at several distances from the soma (**Figure 4.4A**).

Previous studies across AD mouse models have found varying degrees of non-specific synaptic loss in the hippocampus^{115,116,309}. To elucidate the circuit specificity of A β - and microglia-dependent synaptic changes, we imaged SR oblique dendrites from EGFP-labeled CA1-to-PFC projection neurons and reconstructed dendritic spines, calculating spine densities (spines/ μ m) for each segment (**Figure 4.3B**). We used dendritic spines as proxies for synapses as virtually all excitatory synapses are formed at spines on CA1 pyramidal cells, and approximately all spines contain a single excitatory synapse^{340,341}. Our measured densities were comparable to those found with array tomography (AT) and by serial section electron microscopy (ssEM) on the same branch types from mouse CA1^{302,338} (**Figure 4.4B**). Significant main and interactive effects were identified from B6 dendrites, with B6 TG control branches having significantly higher spine densities than branches from B6 WT control and WT PLX5622. This effect was absent in B6 TG PLX5622-treated mice (**Figure 4.3C**), suggesting A β -dependent increases in spine densities required microglia. None of these effects were evident on oblique branches from PWK mice regardless of genotype and treatment.

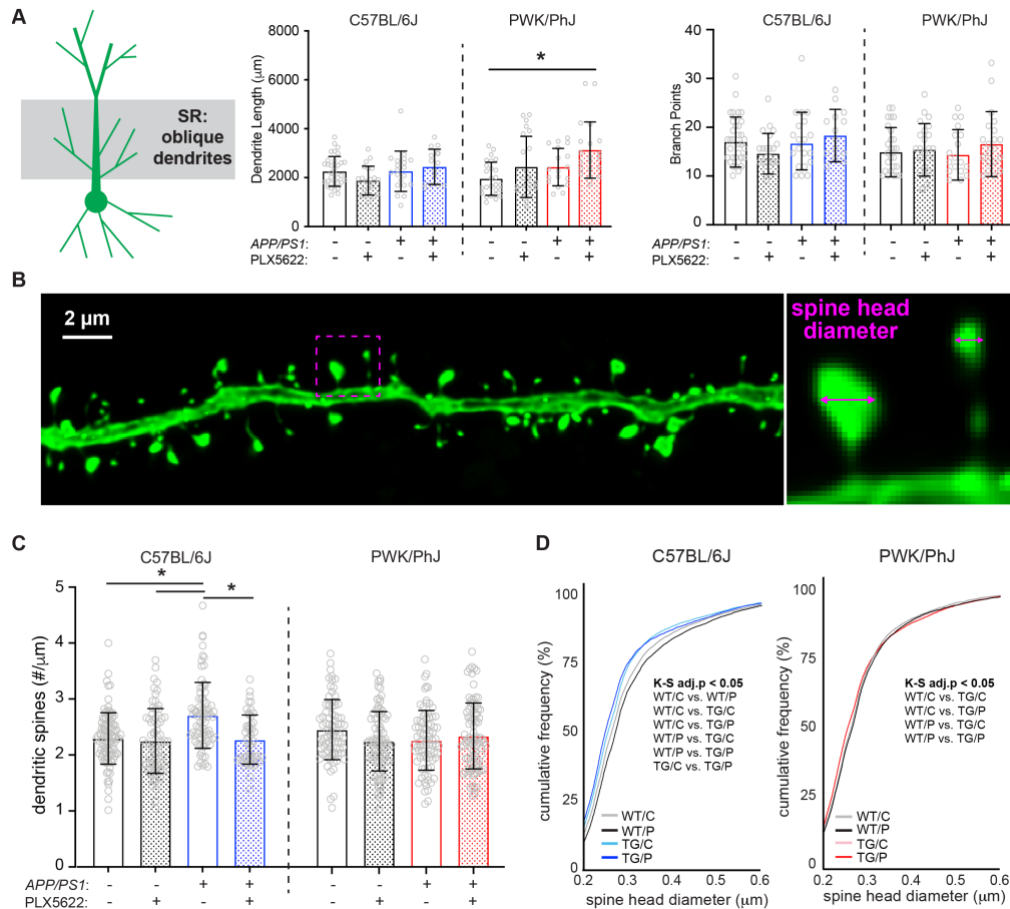


Figure 4.3: Amyloid- and microglia-dependent spine plasticity in oblique branches from B6 but not PWK mice.

(A) Oblique dendritic lengths (left) and branch points (right). Individual data points represent each reconstructed neuron ($n=3-5$ /mouse); error bars are $\pm$ SD; asterisks denote post-hoc ($p<0.05$) after two-way ANOVA and corrections for multiple comparisons. (B) Example deconvolved confocal image of an EGFP+ oblique branch. Spine density was acquired across dendrites (left) and individual spines measured for maximum head diameter (right). (C) Oblique branch spine densities across genotype/treatment groups. Data points represent individual branches ($n=10-15$ /mouse); error bars are $\pm$ SD; asterisks denote comparisons ($p<0.05$) identified between control and PLX5622 groups after two-way ANOVA and corrections for multiple comparisons. (D) Spine head diameter cumulative distributions from B6 (left) and PWK (right). Kolmogorov-Smirnov (K-S) tests were used to evaluate statistical significance, with significant (adj. $p<0.05$) pairwise comparisons reported on the figure. WT/C = Wild-type Control, WT/P = Wild-type PLX5622, TG/C = APP/PS1 Control, TG/P = APP/PS1 PLX5622. Statistical analyses performed on B6 and PWK separately. For (A) and (C) *adjusted $p<0.05$ Bonferroni post-hoc tests.

Synapse morphology is a reliable predictor of synaptic stability and strength^{92,155} so we analyzed the maximum head diameter of each reconstructed spine. Comparison of spine sizes across the B6 groups revealed two prominent changes relative to the spine size distribution of WT control mice: a leftward shift in distribution of B6 TG control and TG PLX5622 compared to B6 WT control – indicating a population dominated by smaller spines, and a rightward shift in B6 WT PLX5622 compared to WT control – indicating a population dominated by larger spines. Like the spine density results, these shifts in spine sizes were noticeably absent across dendrites from PWK mice (**Figure 4.3D**). The opposing shifts in spine sizes across B6 mice was also evident in quartile-based density analyses (**Figure 4.4C**). The same quartile analysis from PWK mice revealed no differences, indicating a remarkable strain-specific stability of spine morphology to A β pathology or microglia depletion.

Basal branches in SO receive the same afferents from hippocampal area CA3 as the apical oblique branches in SR. The changes observed in apical oblique dendrites and dendritic spines were recapitulated in analyses of the basal dendrites in SO from these same projection neurons (**Figure 4.5**), including the relative maintenance of dendritic architecture, the TG-dependent increase in spine densities in B6 mice, and the morphological shift to smaller spine sizes between B6 WT control and TG control mice. Like the oblique dendrites, none of these effects were evident in basal dendrites from PWK mice. These results collectively show that B6 mice are vulnerable to A β -dependent changes in oblique (and to a lesser degree basal) spine density and morphology, while spines on dendrites from PWK mice appear resilient to A β pathology (**Figure 4.5**).

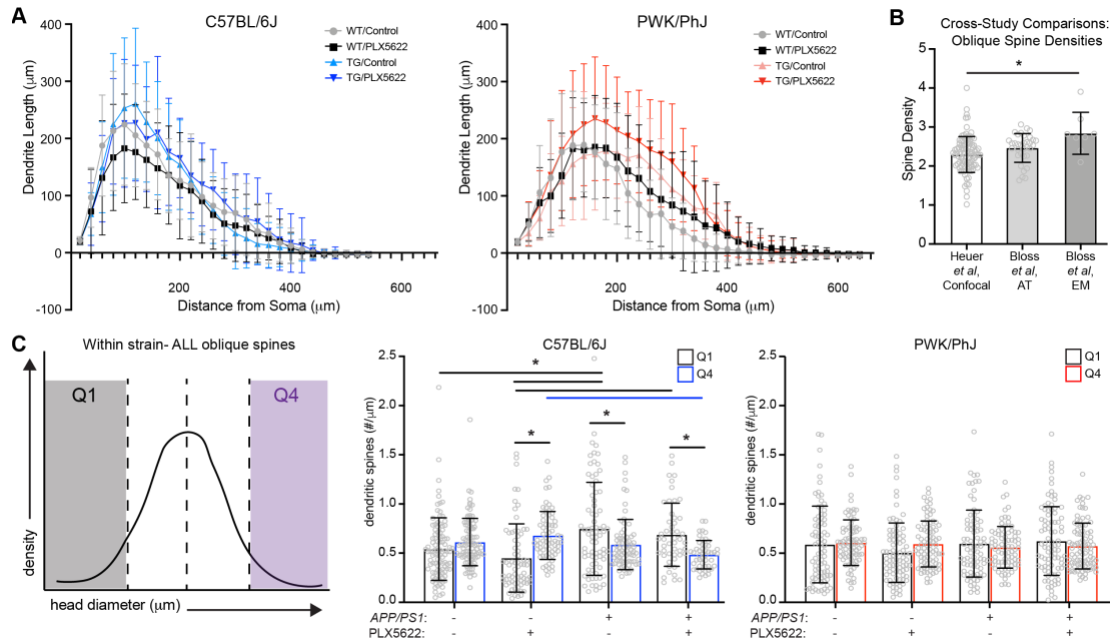


Figure 4.4: Analyses of oblique dendrite structural organization and spine size analysis, related to Figure 4.3.

(A) Sholl analysis of oblique dendrites reporting dendritic length (μm) in 20μm concentric distances from the soma origin. Data presented as mean ± SD of reconstructed neurons (n=3-5/mouse). Two-way ANOVA of dendrite length identify significant ($p<0.05$) genotype effect 120μm and 160-200μm away from the neuronal soma in B6. In PWK significant treatment identified at 220μm and 300-340μm, significant genotype 220-380μm, and significant interaction at 100μm, 380μm and 480-500μm from soma. **(B)** Comparisons of oblique spine densities acquired from B6 WT/+ chow animals from the current study to array tomography (AT) and serial section electron microscopy (ssEM) oblique densities acquired from previous studies^{28,35} with young, B6 mice. Data on graph is presented as mean ± SD, with data points representing measures from individual branches. One-way ANOVA identified significant ($p<0.05$) effect with $F = 6.578$. **(C)** Quartile-based analyses of oblique spine head diameters. All oblique spines within each strain were divided into quartiles based on head diameter (μm). The smallest spines assigned to the first quartile (black, Q1) and the largest spines assigned to the fourth quartile (blue/red, Q4) were identified and reassigned back to originating dendrite. Spine densities (spines/μm) for Q1 and Q4 spines were calculated separately. Data points represent individual oblique branches. Two-way ANOVA within Q1 identified significant ($p<0.05$) genotype effect, and within Q4 identified significant genotype and interaction. One-way ANOVA across quartiles identified significant effect in B6 ($F=7.582$) with no effect in PWK. All data in bar graphs presented at mean ± SD, with individual measures plotted as grey points. Statistical analyses (unless noted otherwise) performed on B6 and PWK mouse strains separately. For (B) and (C) *adjusted $p<0.05$ Bonferroni post-hoc tests.

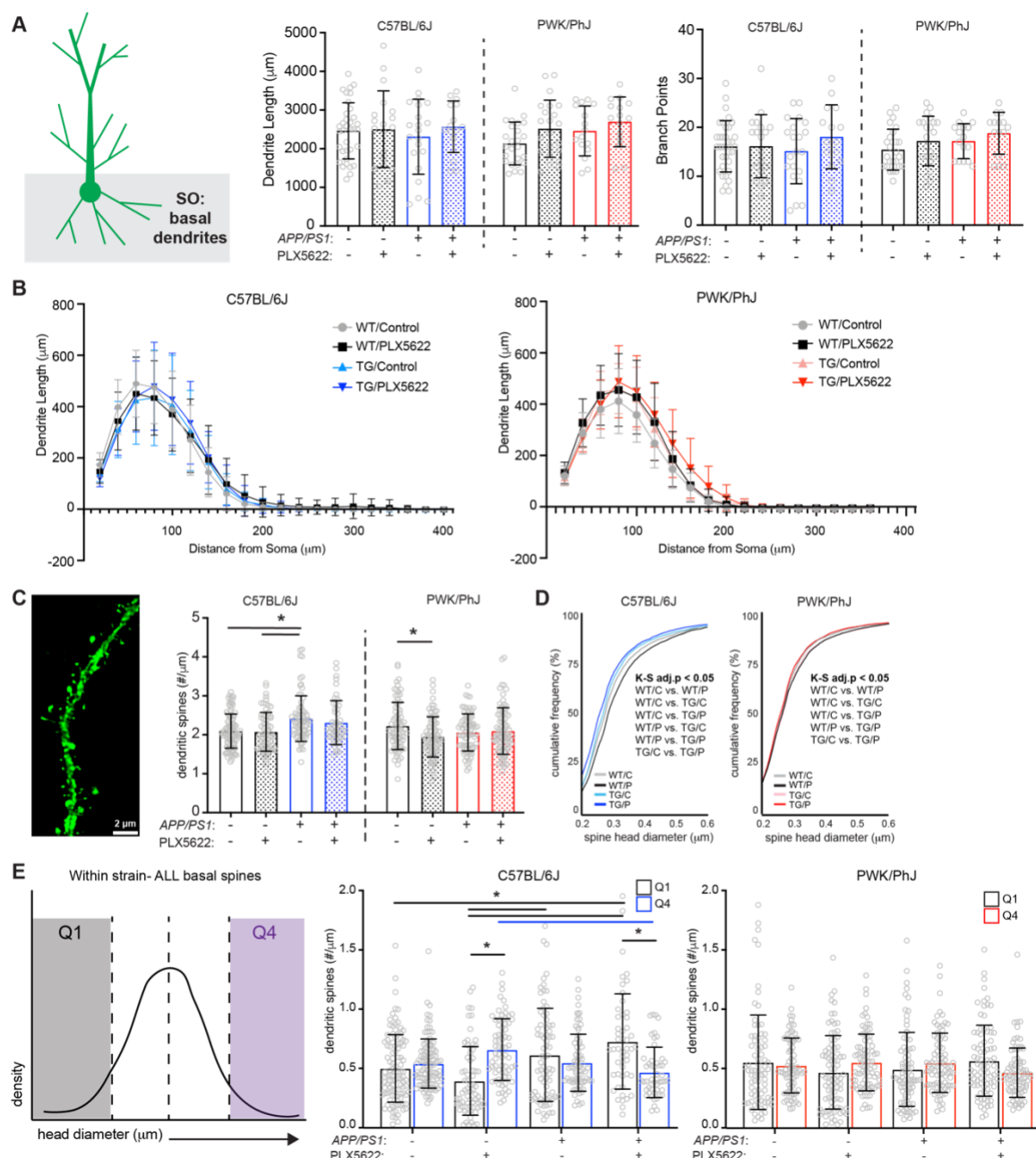


Figure 4.5: Basal dendritic structure, spine density and spine size, related to Figure 4.4.

(A) Basal dendritic reconstruction analyses quantifying total dendritic length (left) and number of branch points (right) across the dendritic tree. GFP+ dendrites were manually reconstructed from confocal images acquired at 40X magnification. Reconstructions were assigned an origin at the neuronal soma and traced all basal branches until point of termination. Individual data points represent each reconstructed neuron ($n=3-5/\text{mouse}$). Two-way ANOVA for total dendrite length identify significant ($p<0.05$) treatment effects for PWK only. **(B)** Sholl analysis of basal dendrites reporting dendritic length (μm) in $20\mu\text{m}$ concentric distances from the origin set at the neuronal soma. Data on graph is presented as mean $\pm$ SD of reconstructed neurons. Two-way ANOVA of dendrite length identify significant ($p<0.05$) treatment effect at $20\mu\text{m}$, and genotype effect $20-40\mu\text{m}$ from

neuronal soma in B6. In PWK significant treatment at 120-200 μ m, genotype at 140-240 μ m, and interaction at 60 μ m and 180 μ m from soma. **(C)** Image of a basal EGFP+ branch (left), and results of basal spine densities (spines/ μ m; center) across genotype/treatment groups. Data points represent individual branches (right, n=10-15/mouse). Two-way ANOVA identify significant ($p<0.05$) genotype in B6, and significant treatment effect and interaction in PWK. **(D)** Cumulative distributions of spine head diameters (μ m) for basal spines across genotype/treatment groups, separated by strain. Statistical results from Kolmogorov-Smirnov (K-S) tests for differences in cumulative distributions, with significant (adj. $p<0.05$) pairwise comparisons reported on the figure. WT/C = Wild-type Control, WT/P = Wild-type PLX5622, TG/C = *APP/PS1* Control, TG/P = *APP/PS1* PLX5622. **(E)** Quartile-based analysis of basal spine head diameters (performed as in **Figure 4.4C**). Two-way ANOVA in B6 identified significant ($p<0.05$) genotype effects and interaction in both Q1 and Q4, and in PWK identified significant interactions in both Q1 and Q4. One-way ANOVA across quartiles identified significant effect in B6 ($F=8.564$) with no effect in PWK. All data in bar graphs presented at mean $\pm$ SD, with individual measures plotted as grey points. Statistical analyses (unless noted otherwise) performed on B6 and PWK mouse strains separately. For (A), (C), (E) *adjusted $p<0.05$ Bonferroni post-hoc tests.

4.3.3 Differential patterns of spine loss or spine remodeling on the distal CA1 tuft dendrites

The distal dendrites of CA1 pyramidal cells receive synaptic input from neurons in the entorhinal cortex (EC), and these synapses show distinct morpho-molecular properties relative to synapses in SR and SO³⁴². Although these inputs are more strongly filtered than those made onto the basal or oblique branches, they nevertheless are critical contributors to feature-selective firing of CA1 pyramidal cells^{343,344}. Like the maintenance of dendritic morphology in SR and SO, we found no significant effects on dendritic length across B6 and PWK groups except for a significant treatment effect on B6 branch points (**Figure 4.6A**). Additional post-hoc and Sholl analyses suggested no two groups differed (**Figure 4.7A**).

Spines on tuft dendrites are lower in density but larger than those on basal or oblique (**Figure 4.6B**)³³⁵, suggesting these spines may be more stable as a population than those found on proximal SR/SO branches. Our measured tuft spine densities from B6 WT control mice recapitulated those obtained from AT and ssEM reconstructions from B6 mice (**Figure 4.7B**). In contrast to the effects observed in SR and SO, we observed no significant effects on spine densities from dendrites of B6 mice irrespective of genotype or treatment. In PWK mice, we found significant genotype and interactive effects, with TG control and TG PLX5622 mice exhibiting significantly lower spine densities than WT counterparts (**Figure 4.6C**).

We next analyzed spine morphologies on these branches and observed a similar pattern of spine redistributions as those found on more proximal branches in B6 and PWK mice. Spines on branches from B6 TG control mice exhibited a leftward shift, indicating A β resulted in a population of smaller spines, while B6 WT PLX5622 branches shifted rightward, indicating microglia depletion resulted in a population of larger spines. Like the results from SR and SO, the experimental PWK groups showed no significant

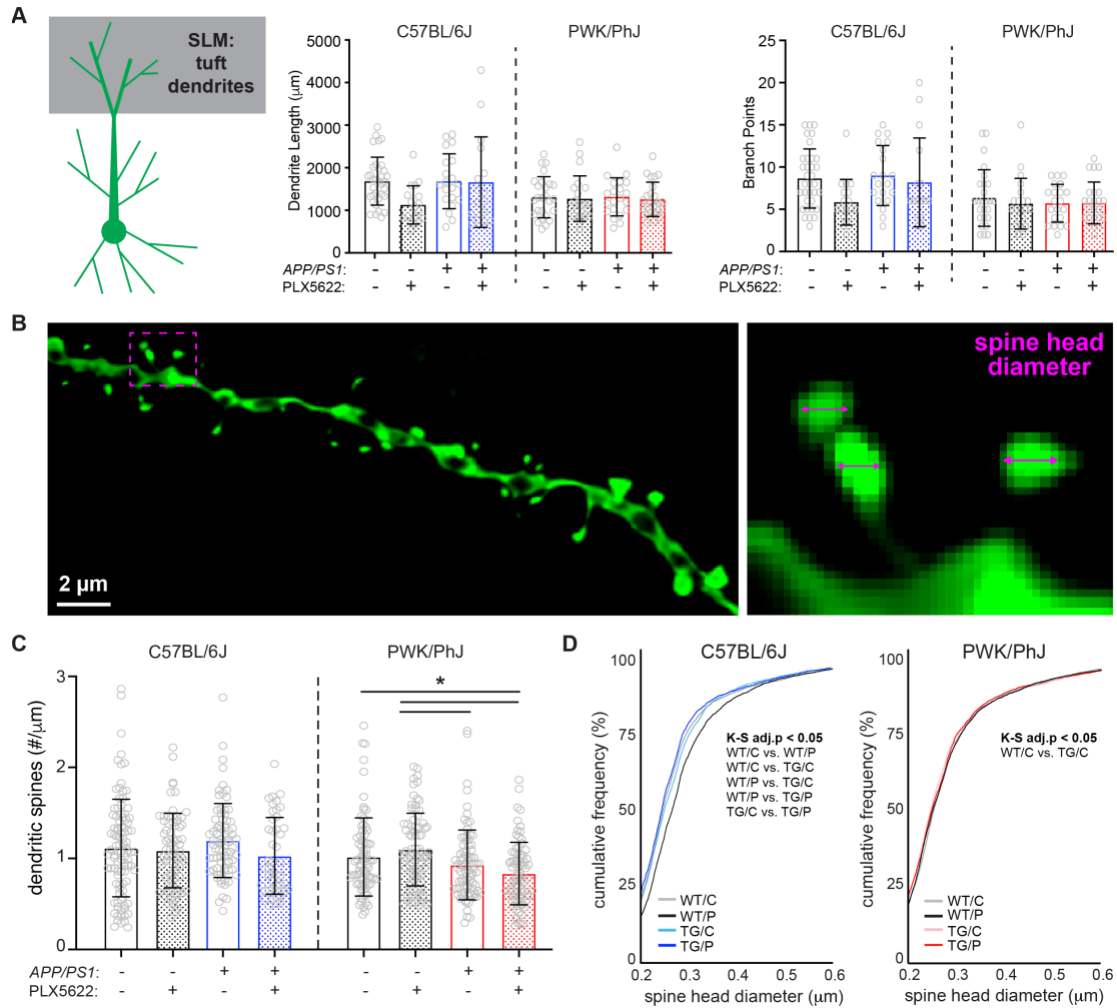


Figure 4.6: Differential regulation of spine density and morphology by amyloid on tuft branches from B6 and PWK *APP/PS1* mice.

(A) Tuft dendritic lengths (left) and branch points (right). Individual data points represent each reconstructed neuron ($n=3-5/\text{mouse}$); error bars are $\pm$ SD; asterisks denote post-hoc ($p < 0.05$) after two-way ANOVA and corrections for multiple comparisons. **(B)** Example deconvolved confocal image of an EGFP+ tuft branch segment. Spine density was acquired across dendrites (left) and individual spines measured for maximum head diameter (right). **(C)** Tuft branch spine densities across genotype/treatment groups. Data points represent individual branches ($n=10-15/\text{mouse}$); error bars are $\pm$ SD; asterisks denote comparisons ($p < 0.05$) identified between PLX5622 and control diet groups after two-way ANOVA and corrections for multiple comparisons. **(D)** Spine head diameter cumulative distributions from B6 (left) and PWK (right). K-S tests were used to evaluate statistical differences, with significant (adj. $p < 0.05$) pairwise comparisons reported on the figure. WT/C = Wild-type Control, WT/P = Wild-type PLX5622, TG/C = *APP/PS1* Control, TG/P = *APP/PS1* PLX5622. Statistical analyses performed on B6 and PWK separately. For (A) and (C) *adjusted $p < 0.05$ Bonferroni post-hoc tests.

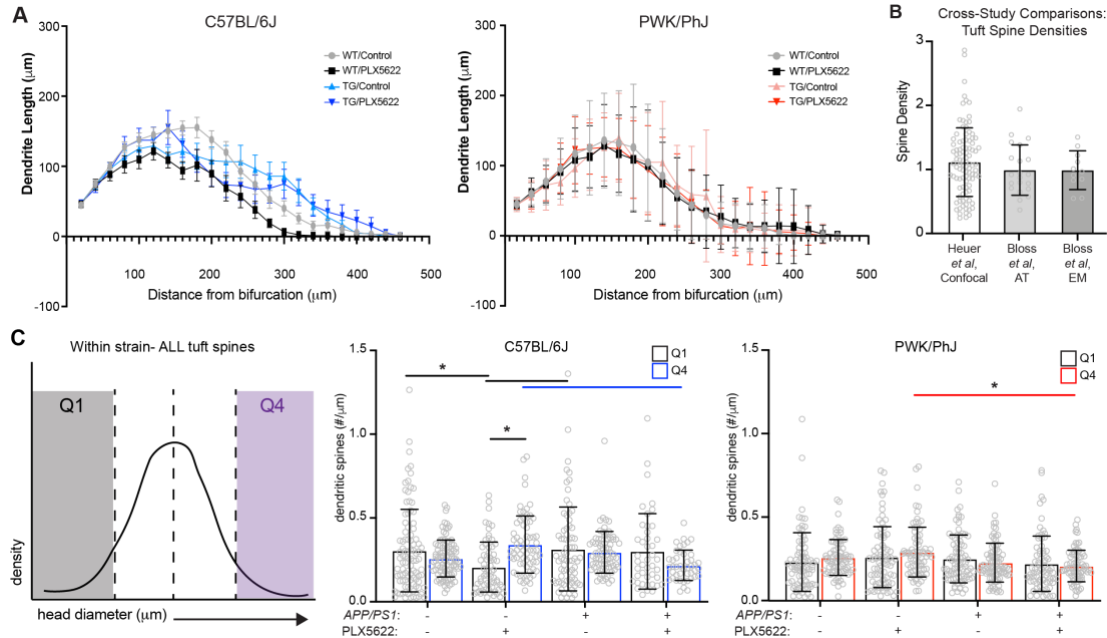


Figure 4.7: Analyses of tuft dendritic structure and spine sizes, related to Figure 4.6. (A) Sholl analysis of tuft dendrites reporting dendritic length (μm) in $20\mu\text{m}$ concentric distances from the origin set at the main bifurcation of the primary apical branch. Data on graph is presented as mean $\pm$ SD of reconstructed neurons ($n=3-5/\text{mouse}$). Two-way ANOVA of dendrite length identify significant ($p<0.05$) genotype effect $220\mu\text{m}$, treatment effect $280-380\mu\text{m}$, and interaction $140-160\mu\text{m}$ from main bifurcation in B6 only. **(B)** Cross-study comparisons of tuft spine densities acquired from B6 WT/+ chow animals from the current study (using confocal microscopy), AT and ssEM tuft densities acquired from young, B6 mice^{28,35}. Data on graph is presented as mean $\pm$ SD, with data points representing measures from individual branches. One-way ANOVA identified no significant effect. **(C)** Quartile-based analysis of tuft spine head diameters (as in **Figure 4.4C**). Two-way ANOVA within Q1 identified significant ($p<0.05$) treatment effect, and within Q4 identified significant genotype and interaction in B6, and significant genotype effect within Q4 in PWK. One-way ANOVA across quartiles identified significant effects in both B6 ($F=4.419$) and PWK ($F=2.666$). All data in bar graphs presented at mean $\pm$ SD, with individual measures plotted as grey points. Statistical analyses (unless noted otherwise) performed on B6 and PWK mouse strains separately. For (C) *adjusted $p<0.05$ Bonferroni post-hoc tests.

size redistributions (**Figure 4.6D**), further supporting the resilience of this strain to A β or microglia activity. Quartile-based analysis further validated these population shifts in B6 but not PWK (**Figure 4.7C**). The disassociation between microglia-independent forms of amyloid-induced spine remodeling in B6, and spine loss in PWK, further supports the

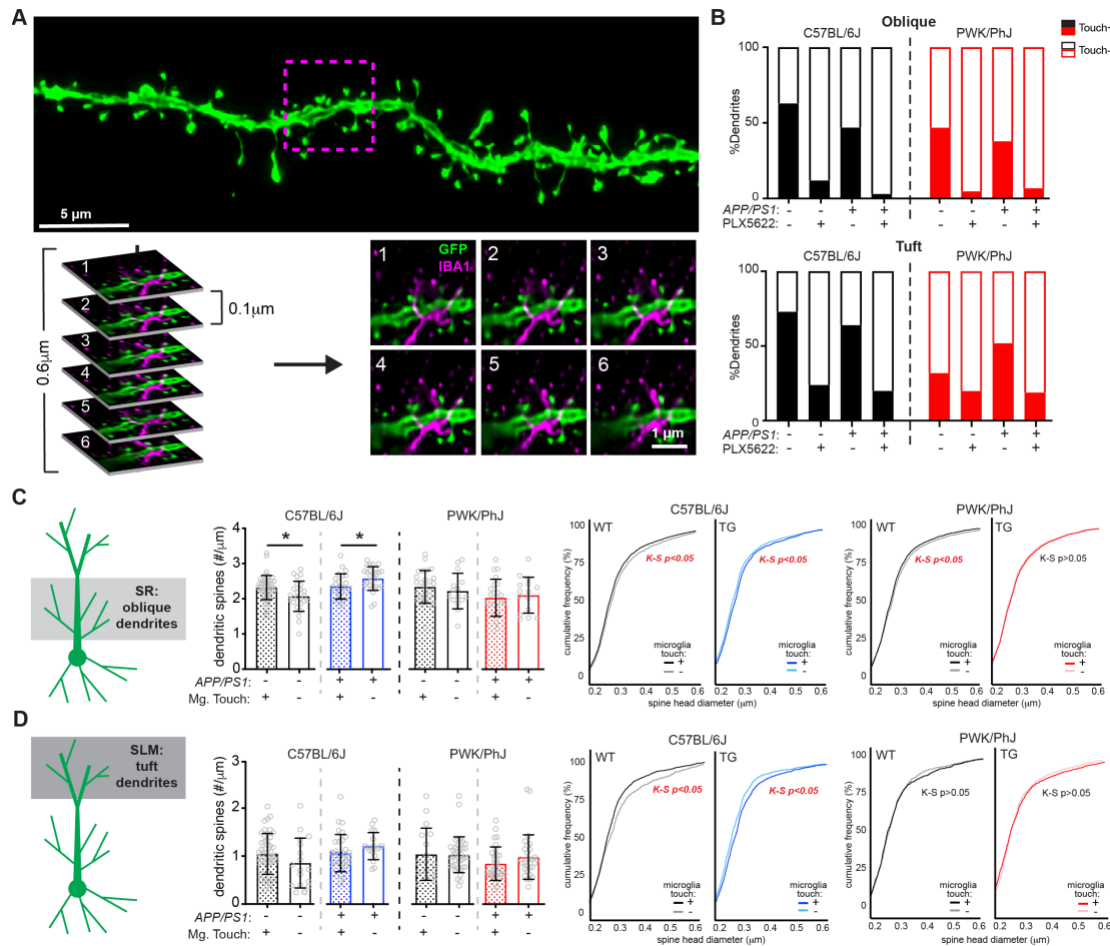
notion that these two strains are inherently different in their neuronal responses to A β pathology.

4.3.4 Microglia-dendrite interactions influence spine density and size over large spatial scales

Microglia can influence neuronal synapses broadly through the release of diffusible factors, or locally at points of microglia-dendrite interactions^{87,127,193}. To establish if the latter scenario was evident within our data, we examined spine data across dendritic segments that did (Touch+) or did not (Touch-) physically interact with IBA1+ microglia (**Figure 4.8A**). Approximately 50% of dendrites from control diet mice were Touch-, whereas approximately 90% of dendrites from PLX5622 mice were Touch- (**Figure 4.8B**), providing a highly local estimate of depletion at our sampled branches. Given the sparsity of Touch+ dendrites in PLX5622 groups, analyses of microglia touch-based effects on spine density and size were only performed across control diet groups.

In B6 WT mice, Touch+ oblique dendrites exhibited significantly higher spine densities compared to Touch-. Conversely, in B6 TG mice, Touch+ oblique dendrites showed significantly lower spine densities compared to Touch- (**Figure 4.8C**, left). These data suggest that in the absence of A β , microglia-dendrite interactions promote higher rates of synaptic connectivity, whereas microglia exposed to A β promote synaptic loss when contacting dendrites. Both effects were absent on dendrites from PWK mice, emphasizing that genetic context controls how microglia regulate dendritic spines. Spines on Touch+ oblique dendrites from B6 WT mice were significantly smaller than those from Touch-, whereas spines on B6 TG Touch+ branches were significantly larger than Touch-. These patterns of microglia touch-dependent spine size changes were present on dendrites from PWK WT mice, but absent in PWK TG mice (**Figure 4.8C**,

right). Thus, in terms of spine density and morphology, these data show that, at least in the specific circuit tested here, microglia-dendrite interactions regulate dendritic spines differently across B6 and PWK mice during A β plaque deposition.



Analysis of Touch+ and Touch- dendrites from the distal tuft compartment revealed no differences in dendritic spine density across B6 and PWK mice, regardless of genotype (**Figure 4.8D**, left). Like oblique branches, spines from B6 WT Touch+ tuft spines were significantly smaller than Touch-, while spines from B6 TG Touch+ were significantly larger than Touch-. Therefore, in B6 mice, microglia play opposing roles on spine morphology in healthy versus A β conditions. In contrast, PWK tuft spines exhibited no size differences between Touch+ and Touch- dendrites, regardless of genotype (**Figure 4.8D**, right).

Functional interactions among synapses can be highly localized within dendrites. For example, the induction of structural plasticity at one spine can lower the threshold for plasticity at neighboring spines within a restricted 5-10 μ m window^{284,345}. Therefore, we wondered if the effect of microglia Touch+ would be enhanced at the microglia-dendrite interaction point relative to adjacent locations on the same segment. Within Touch+ dendrites, we identified the point of microglia-dendrite interaction, and measured densities and head diameters of spines 5 μ m in each direction from the interaction point (proximal) and compared this to measures taken from adjacent 10 μ m-long dendritic section(s) (distal) (**Figure 4.9A**) from the same branch. Surprisingly, we did not identify significant differences in spine density and size between proximal and distal regions, regardless of strain or genotype (**Figure 4.9B**). The same analysis of distal tuft dendrites revealed a subtle yet significant increase in spine density in PWK WT animals at proximal compared to distal regions, but equivalent spine sizes across locations. Conversely, we found no differences in spine density at proximal versus distal regions in PWK TG mice but found that proximal spines were smaller than distal spines (**Figure 4.9C**). Thus, physical interactions between microglia and dendrites impact synaptic density and morphology over relatively large (mean segment length was $42 \pm 4.9\mu$ m)

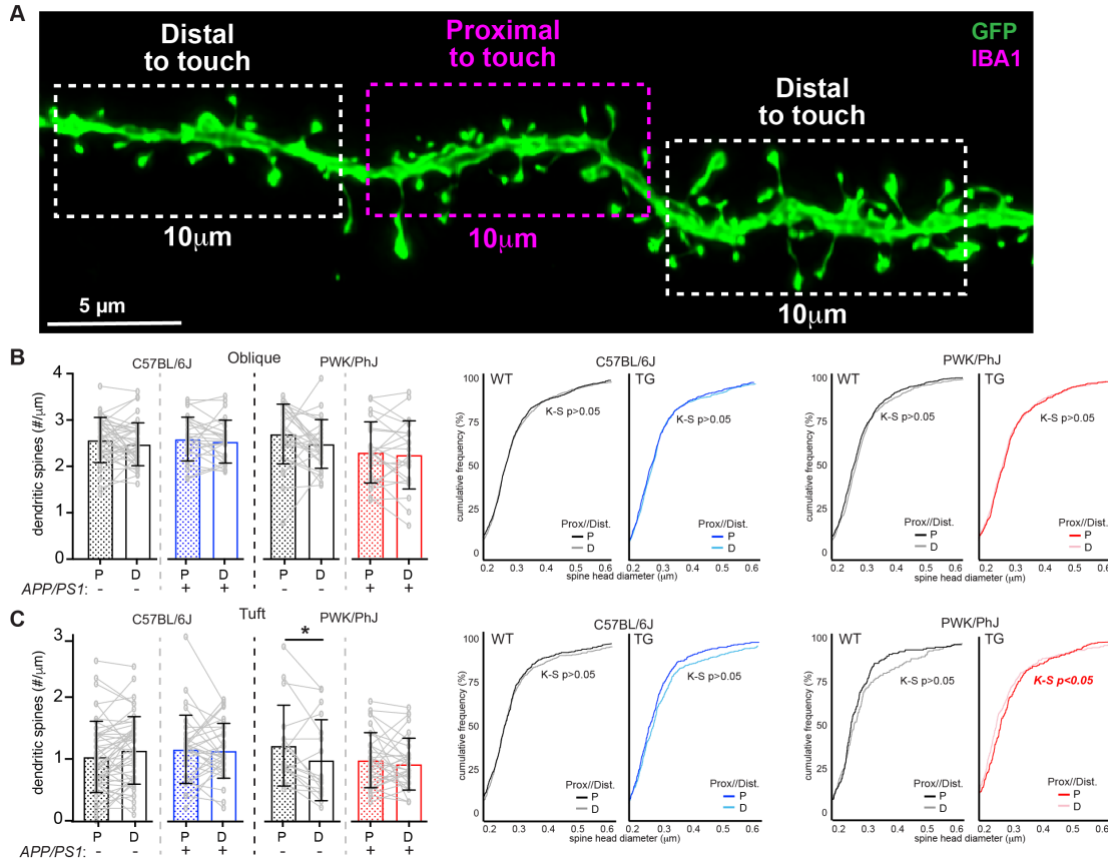


Figure 4.9: Microglia-dendrite interactions do not selectively shape spines at the point of contact, related to Figure 4.8.

(A) Image of the dendrite depicted in Figure 4.8A, with outlines depicting regions that are proximal and distal to a microglia-dendrite interaction (Touch+). 10μm of each type of segment for each dendrite with a microglia contact were quantified for spine density and head diameter. (B) Analysis of spine density (left) and head diameter (right) of Touch+ oblique branches, comparing region of the dendrite proximal versus distal to the microglia contact. Spine density data points represent individual branches ($n \leq 15$ mouse). Paired t-tests were performed to compare spine densities between proximal and distal branch segments within each strain/genotype group. (C) Identical analysis to (B) for tuft dendrites. For (B) and (C) $*p < 0.05$, paired t-test. Cumulative distributions were statistically analyzed with Kolmogorov-Smirnov (K-S) tests.

spatial scales in B6 mice. Moreover, even when restricted to locations on branches where microglia physically interact with dendrites, responses in B6 mice still appear fundamentally different from PWK mice. Given the literature demonstrating individual branches act as computational subunits^{346–348} and as important structures for information storage^{349,350}, the different synaptic vulnerabilities to amyloid observed between B6 and

PWK mice likely underlie the functional cognitive resilience to amyloid we have found between these strains previously⁵³.

4.4 Discussion

We sought to determine how microglia regulate synaptic connectivity on CA1 pyramidal neurons during exponential A β plaque deposition, and whether this phenomenon appears uniformly across genetically diverse contexts. Our analysis was restricted to CA1-to-PFC projecting pyramidal cells because they are the primary projection circuit to ventral PFC¹⁶⁷, comprise an important pathway gating the progression of AD¹⁶⁸, and allowed for stringent comparisons within a single projection class across diverse mice. We took advantage of the same transgenic A β driver (*APP/PS1*) across genetically distinct B6 and PWK mouse strains and generating high-resolution reconstructions of ~145,000 dendritic spine synapses. PLX5622 administration produced equivalent rates of CA1 A β plaque deposition and microglia depletion across B6 and PWK mice, yet robust differences remained evident in the synaptic responses across the strains (**Table 4.1**). These differences persisted even when accounting for physical microglia-dendrite interactions at the level of individual branches (**Table 4.2**). Such a result strongly supports incorporating genetic diversity into mouse models of AD to faithfully recapitulate resilience, resistance, and susceptibility that are observed in the human patient population. Mechanistically, it shows the PWK strain harbors resilience factors that can be identified in future genetic mapping studies.

In B6 mice, CA1-to-PFC projection neurons showed significant changes in spine density and morphology on oblique branches in a microglia-dependent manner. More broadly, across all dendritic domains we found that *APP/PS1* and PLX5622 induced differential remodeling of spine sizes such that the overall spine population consisted of

smaller (less stable^{92,335,351}) synapses in the presence of A β plaque pathology, yet larger (more stable^{92,335,351}) synapses in the absence of microglia. At a finer granularity, our results showed these changes in the B6 mice depend on whether physical interactions existed between microglial processes and individual pyramidal cell dendrites. Conversely, CA1-to-PFC neurons from PWK mice were resistant to structural changes induced by A β pathology and/or microglia contacts. This PWK neuronal resilience and B6 susceptibility paralleled results we reported previously that PWK mice fail to exhibit cognitive deficits (measured on a delayed spatial behavioral assay) in the presence of the *APP/PS1* transgene. These results contrasted with a decrease in successful performance in *APP/PS1* mice on the B6 strain background⁵³. It is possible that PWK and B6 could exhibit divergence on more than one cognitive assay, indicative of a much broader form of resilience across multiple cognitive domains. As the wildness score (measure of jumping, escape, struggle, squeaking and biting) of B6 falls around 0.27 but PWK around 1.35, it was important to leverage behavioral assays that would carry little bias towards or against behaviors selective to or against wild-derived mice³⁵². Therefore, tasks that require repeated handling and aversive stimuli (such as contextual fear conditioning) may not properly capture memory performance from wild-derived strains. Future work can leverage more in-depth behavioral assays to identify the extent of cognitive resilience across PWK and B6 now that we have identified the PWK as a strain with cognitive and synaptic resilience to A β pathology.

While our results agree with past work that reports that early dynamics of synaptic changes in AD patients and mouse models may be subtle^{309,339,353,354}, more extreme rates of excitatory synapse loss in response to amyloid-driving AD transgenes have been reported^{115,116}. The differences between these studies and ours are likely due to several experimental factors (e.g., age, amyloid-driving transgene, methods estimating

synaptic connections, and neurons examined). Being the first to examine A β - and microglia-dependent synaptic changes on specific projection neurons, it is possible that the CA1-to-PFC projection pathway we examined here shows a different pattern of synaptic responses than those on neurons chosen at random^{116,339} or synapses sampled randomly from the neuronal population as a whole^{115,309}. Conversely, the magnitude of PLX5622-induced microglia depletion was lower than previously reported¹³⁹. We view this as a feature rather than a detriment, as this lowered depletion allowed us to measure microglia-dependent neuronal effects without influencing A β plaque pathology across *APP/PS1* mice.

One uniform feature across B6 and PWK mice was the heterogeneous laminar distribution of microglia within area CA1. The higher density of microglia in SLM was associated with elevated A β plaque burden which correlated with lower rates of PLX5622-mediated depletion, suggesting microglia in different hippocampal lamina may perform specific functions or belong to different transcriptionally-defined states¹³⁰. Microglia states have gained increased interest with the initial discovery of disease-associated microglia (DAM) as the primary amyloid-induced state¹²² and interferon-responding microglia (IRM) as the primary aging-induced state¹²⁹. Interestingly, we have found that B6.*APP/PS1* and PWK.*APP/PS1* female mice develop different proportions of these two microglia states⁵⁴. Whether the differing susceptibility/resilience to CA1-to-PFC synaptic changes during A β plaque deposition across B6 and PWK *APP/PS1* mice seen here can be ascribed to specific microglia states should be determined through state-specific manipulation approaches.

The high-resolution assays used here, combined with the assessment of two genetically distinct mouse strains and two diet conditions, necessitated the use of only female mice in this study. Sex differences are important factors for dictating AD

heterogeneity³⁵⁵, and have been reported in AD models including wild-derived mouse strains⁵³. Similarly, we employed the *APP/PS1* transgene that is most relevant to A β deposition seen in familial AD^{40,41}, but the approach developed here can be applied more broadly to determine microglia-dependent synaptic changes across mouse models that are relevant to LOAD^{30,356,357}. Despite these caveats, these are the first data to show that genetic context determines whether microglia are recruited to modify synapses in response to amyloid.

Our data suggest PWK mice exhibit resilience that appears to stem from factors independent of microglia, including contributions of other non-neuronal cell types such as astrocytes, or via intrinsic neuronal adaptations. Recent studies in the Tau^{P301S} mouse model provide evidence suggesting that astrocytes (rather than microglia) prune excitatory synapses³⁵⁸. Since astrocytes are known to facilitate synaptic connections, they are well-positioned to promote synaptic adaptations in PWK mice under AD-like conditions of amyloid deposition. The second possibility is that PWK neurons upregulate factors that produce adaptive responses to A β deposition. A recent study has shown that a patient exhibiting resilience to autosomal dominant AD who also carried a putative protective variant in *RELN*, a gene with importance in neuronal development²⁴. It could be that neurons from PWK mice, in an analogous manner to this resilient individual, upregulate neurodevelopmental pathways that effectively protect neurons from the damage occurring during amyloid deposition. Additional large-scale unsupervised analyses leveraging single cell technologies will lead to a greater identification of these factors and of additional cell type(s) outside of microglia driving resilience in PWK neurons.

Identification of the genetic, cellular, and circuit-specific mechanisms of the resilience displayed in PWK mice could reveal novel therapeutic targets to prevent AD progression

and promote early cognitive resilience across all patients. More broadly, these results strongly suggest that the mechanisms underlying synaptic changes in early AD vary across genetically diverse individuals. From a translational perspective, such a result suggests a low response rate to “one-size-fits-all” approaches to therapeutic interventions aimed at restoring synaptic structure and function during AD progression. Discovery of these neuronal resilience mechanisms could serve as a rational entry point towards precision therapeutic strategies in genetically defined subsets of AD patients.

Table 4.1 Summary of observed spine responses to A β and microglia depletion in CA1-to-PFC projection neurons.

	Experimental Groups	Experimental measures	
		Density	Head Diameter
C57BL/6J Basal	WT/+ Control vs. WT/+ PLX5622	--	↑
	WT/+ Control vs. TG/+ Control	↑	↓
	TG/+ Control vs. TG/+ PLX5622	--	↓
C57BL/6J Oblique	WT/+ Control vs. WT/+ PLX5622	--	↑
	WT/+ Control vs. TG/+ Control	↑	↓
	TG/+ Control vs. TG/+ PLX5622	↓	↑
C57BL/6J Tuft	WT/+ Control vs. WT/+ PLX5622	--	↑
	WT/+ Control vs. TG/+ Control	--	↓
	TG/+ Control vs. TG/+ PLX5622	--	↓
PWK/PhJ Basal	WT/+ Control vs. WT/+ PLX5622	↓	↑ (small ES)
	WT/+ Control vs. TG/+ Control	--	↑ (small ES)
	TG/+ Control vs. TG/+ PLX5622	--	↓ (small ES)
PWK/PhJ Oblique	WT/+ Control vs. WT/+ PLX5622	--	--
	WT/+ Control vs. TG/+ Control	--	↓ (small ES)
	TG/+ Control vs. TG/+ PLX5622	--	--
PWK/PhJ Tuft	WT/+ Control vs. WT/+ PLX5622	--	--
	WT/+ Control vs. TG/+ Control	↓	↓ (small ES)
	TG/+ Control vs. TG/+ PLX5622	--	--

Table 4.2 Summary of observed spine responses to microglia contacts across A β conditions in CA1-to-PFC projection neurons.

	Experimental Groups	Experimental measures	
		Density	Head Diameter
C57BL/6J Oblique	WT/+ Control: Touch+ vs. Touch-	↑	↓
	TG/+ Control: Touch+ vs. Touch-	↓	↑
C57BL/6J Tuft	WT/+ Control: Touch+ vs. Touch-	--	↓
	TG/+ Control: Touch+ vs. Touch-	--	↑
PWK/PhJ Oblique	WT/+ Control: Touch+ vs. Touch-	--	↓
	TG/+ Control: Touch+ vs. Touch-	--	--
PWK/PhJ Tuft	WT/+ Control: Touch+ vs. Touch-	--	--
	TG/+ Control: Touch+ vs. Touch-	--	--

4.5 Acknowledgements

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4.6 Author contributions

S.E.H., E.B.B. and G.R.H. designed the study, with input from K.D.O. and K.C.G. A.A.H. and K.J.K. generated mouse cohorts and assisted with animal harvests and tissue collection. S.E.H. maintained mouse cohorts, performed all associated experiments, collected, and analyzed final data. E.B.B. provided training in intracranial injections, confocal microscopy imaging of dendrites and dendritic spines, and NeuronStudio analysis. E.B.B. and G.R.H. advised on all data analysis. K.D.O. and K.C.G. advised on data interpretation and manuscript preparation. S.E.H., E.B.B. and G.R.H. wrote the manuscript. All authors approved the final version.

Chapter 5: Discussion

5.1 Summary of thesis work

Many believe that the disconnect between AD model systems and clinical success has been due to the lack of translatability of traditional mouse models²²⁵. Mouse genetic diversity has been largely ignored in AD mouse models, which could be a contributing factor to the limitations of current models. Being that AD is heterogenous in nature, governed by multiple factors including epistatic genetic interactions that create susceptibility and resilience to disease severity^{25,127,331}, the findings from my thesis argue that mouse models should recapitulate human genetic complexity to improve preclinical studies.

Throughout this thesis I leveraged genetically diverse mouse models to study factors that promote healthy aging and disease resilience. First, I employed a population genetics approach to prioritize biological pathways and cell-types that were influential at predicting cognitive outcomes in AD (**Chapter 2**). I then utilized the wild-derived PWK mouse strain that exhibits cognitive resilience and resistance to FAD-causing mutations and aging, respectively, to perform more high-resolution imaging analyses. Since AD is a disease that affects aged individuals, I investigated how PWK and B6 mice synaptically deteriorate with age across excitatory neuronal circuits known to be vulnerable (PFC-to-RE) and resistant (CA1-to-PFC) to age-related synaptic loss (**Chapter 3**). Finally, I tested if PWK exhibited synaptic resilience in CA1-to-PFC neurons in early AD pathological stages (known to be the most critical for disease development), and if this resilience was dependent on microglia (**Chapter 4**). An overview for each of these studies is depicted in **Figure 5.1**.

Overall, the results from my thesis suggest that there are genetic factors that influence 1) how synapses age and are affected by AD-related neuropathologies, and 2) the impact that microglia have on destabilization of neuronal synapses in AD. My results also suggest that additional cell types outside of microglia, such as astrocytes, could be involved in individual disease resilience. My findings supply evidence for continued study of the mechanisms that promote individual resilience (as observed in PWK mice) across human-relevant disease contexts. However, there are additional studies that can be done to support these findings and provide additional more mechanistic insights. In the sections to come I will outline experiments where I could expand on this work in the future to test hypotheses posited from my thesis work.

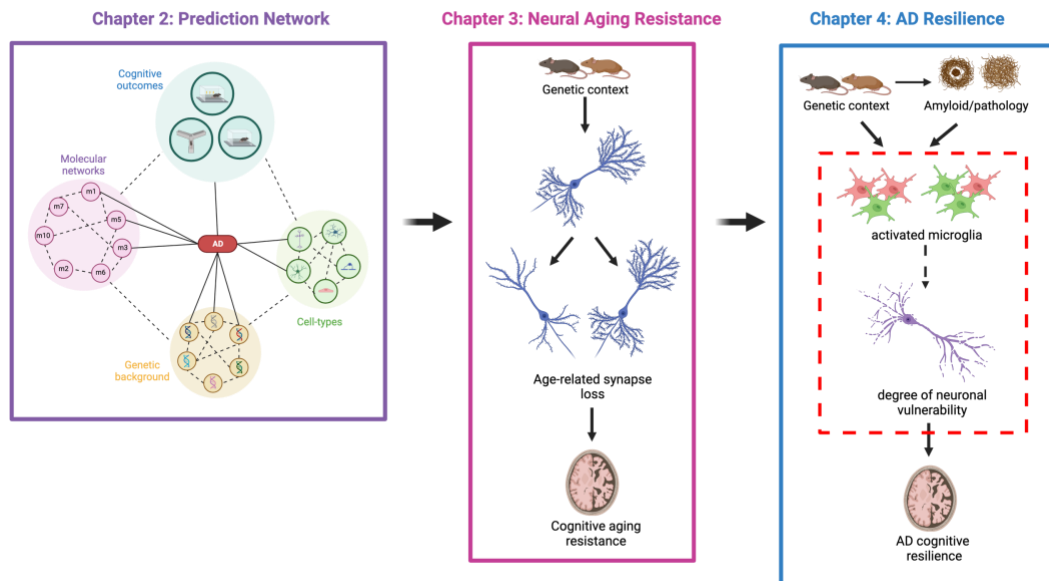


Figure 5.1: Genetic context promotes diversity in microglia-neuron interactions during AD progression. Overview of primary findings from each section of this thesis.

5.2 Multi-omic approaches for identifying candidates to promote healthy brain aging and AD resilience

To evaluate the contribution of genetic diversity to AD mouse models, I leveraged bulk RNAseq data from the AD-BXD mouse population to form modules of co-expressed genes and relate them to individual differences in cognitive performance (**Figure 2.1**). In this scenario, relative transcript abundance in the hippocampus was correlated with cognitive performance on a behavioral task from each mouse in the study. I identified gene modules that correlated with cognitive outcomes and enriched for biological pathways including lipid biosynthesis, viral immunity, synaptic signaling, and neurotransmitter organization (**Figure 2.5**). Comparison of the genes in these modules with previously published datasets²¹³ identified cell-types that these gene modules most likely represented, such as astrocytes, microglia, and neurons. After performing more complex statistical modeling⁷⁰, I generated the hypothesis that genetic variation drives microglia (and pathways within them) to cause individual differences in cognitive outcomes across an AD mouse population (also conserved in human data (**Figures 2.14-2.15**)). However, recent findings regarding the relevance of bulk transcriptomics propose new avenues for this research, including integrative approaches to understand the specific mechanisms that are causing negative cognitive outcomes in AD.

The premise of transcriptomic analyses is based on the biological dogma that DNA is transcribed into RNA which is then translated into protein³⁵⁹. Therefore, transcriptional data (which is relatively easy to acquire) should be a proper representation for the relative activity of pathways in the tissue or cell-type being examined, as greater activity of a pathway would require more biological material in the form of transcripts and proteins to be synthesized. However, recent studies have found there is not a strong correlation between the regulation of transcript and protein abundance in mammalian cells, especially under periods of stress^{360,361}. This is due to the complex nature of

mammalian protein regulation, including splice variants, post-translational modifications, degradation, cleavage, and folding/misfolding. Due to limitations in the ability for transcript to translate to protein abundance (and activity), more comprehensive integrative approaches can be applied to evaluate the activity of biological pathways in a diseased tissue, as well as identify key molecular drivers of cellular dysfunction in aging and AD.

Additional experiments to test findings from **Chapter 2** include leveraging data types that strongly correlate with cellular activity, such as proteomics, lipidomics and metabolomics. Discovery-based mass spectrometry approaches have very recently been applied in human AD brain tissue to identify key proteins differentiating AD from non-AD individuals³⁶². These approaches use tandem mass tag mass spectrometry which evaluates protein abundance based on mass and structure of isolated targets, allowing for a greater number of proteins to be analyzed. This could be leveraged on hippocampal homogenates from AD-BXD mice to evaluate how well changes in transcript abundance across population correspond to protein abundance, and possibly narrow down additional dysregulated candidates through proteomic QTL analysis. In addition to protein abundance, the study of metabolites and lipids in tissue homogenates can allow for evaluating the activity of certain biochemical pathways that are active or inactive and relate to AD outcomes. This is especially relevant to the current findings as the top enriched pathway in one of the modules predicted to drive cognitive outcomes was Lipid Biosynthesis. If lipid biosynthesis processes are dysregulated in poor cognitive performers in the AD-BXD populations, a lipid class could be nominated through lipidomic analyses on AD-BXD hippocampus. Lipid droplets accumulating in microglia from AD patient brains are a novel neuropathology²³⁸, and the evaluation of how the lipids in these droplets relate to higher order cognitive outcomes would provide further evidence for pathways that need to be altered to improve AD cognitive symptoms.

Additionally, the synthesis of lipid profiles with proteomic and metabolomic results would nominate dysregulated proteins that could be driving altered lipid metabolism in susceptible individuals.

An analysis often left out of these integrative approaches, and important for the understanding of protein function, is the study of post-translational proteomic modifications. These modifications are a final indicator of protein activity, as phosphorylation/dephosphorylation can determine if an enzymatic binding site is available to activate a molecular pathway or if a protein has a proper conformation for its needed activity. Phosphoproteomic studies in AD patient cortical brain tissue have identified dysregulated kinase networks that indicate proteins (MAPT, SYN1, SNAP25 to name a few) , cell-types (oligodendrocytes, astrocytes, microglia, neurons), and pathways (synaptic signaling, inflammation) that are dysregulated in the disease³⁶³. These studies have solidified a disconnect between transcript and protein abundance, requiring additional data types for a more comprehensive understanding of how genetic variants alter protein abundance. As many of the proteomic studies discussed here have been conducted in patient tissue, it would be valuable to apply these methods to expand upon findings from this thesis to provide deeper insights, regulated genetically, into the early processes occurring in AD mouse models that can predict cognitive outcomes. Synthesizing new proteomic data with previously used transcriptomic data would allow for the identification of biomarkers that could be used in the transcriptome that would predict more complex, dysregulated protein activity.

5.3 Manipulating microglia states *in vivo*

The model established in **Chapter 2** and tested in **Chapter 4** was that the strain-based differences in microglia (possibly through microglia states, although not specifically tested here)⁵⁴ cause resilience (in *PWK.APP/PS1*) and susceptibility (in

B6.*APP/PS1*) to AD-related synaptic changes. To initially test this, I depleted microglia across both amyloidogenic strains (PWK.*APP/PS1* and B6.*APP/PS1*) with the hypothesis that loss of ‘protective’ microglia in resilient PWK.*APP/PS1* mice would cause destabilization of neuronal synapses whereas depletion of ‘damaging’ microglia in B6.*APP/PS1* would be protective to synaptic health. This was based on previous work finding PWK.*APP/PS1* develop different brain microglia states than to B6.*APP/PS1* mice⁵⁴. As expected, in B6.*APP/PS1* we observed A β - and microglia-dependent changes in CA1 spine dynamics that were also modified by microglia contacting the dendritic branch (**Figure 4.8**). However, CA1 dendritic spines from PWK.*APP/PS1* were completely resilient to A β - and microglia-mediated changes in spine density and size, suggesting that microglia in PWK mice are not as important in protecting neuronal synapses from AD-related neuropathology as initially anticipated. However, while complete microglia depletion didn’t have an effect in PWK mice, the contribution of specific microglia states has still yet to be investigated.

To determine if microglia states drive AD progression, conditional and/or inducible mouse models that utilize Cre recombinase can be leveraged. These models allow for the expression of an inserted DNA fragment based on a chosen promoter, in this case microglia state marker gene (**Figure 5.2**)³⁶⁴. Several current systems have allowed for the manipulation of microglia, such as the model in which Cre-recombinase or GFP is driven by a *Cx3cr1* promoter^{365,366}, a Cre-driven *Csf1r* model that can manipulate gene expression in tissue-resident macrophages³⁶⁷, and, even more specifically, a *Tmem119*-CreERT2 model which drives inducible recombinase expression by the systemic injection of Tamoxifen³⁶⁸. Since *Tmem119* expression has been found to be the most restricted to brain-resident microglia, this system allows for a specific manipulation of target gene expression in microglia⁷⁵. By crossing mice that carry myeloid-specific Cre

recombinase with strains that have loxP sites around genes upregulated in microglia states (e.g. *Itgax*/CD11c for DAM, *Ifitm3* for IRM), a more specific interrogation of the influence of a particular state on AD-related phenotypes such as synaptic health can be completed. Furthermore, now that there is a better understanding of how genomes of wild-derived mice can be manipulated, these Cre/loxP systems can be engineered and studied across multiple genetic backgrounds in a way to gather a better understanding of individuals that develop differing states in A β pathological progression. This would allow for a more specified paradigm to understand the contribution of microglia states as damaging or protective to neuronal health, as well as how they could contribute to higher-order measures including cognition.

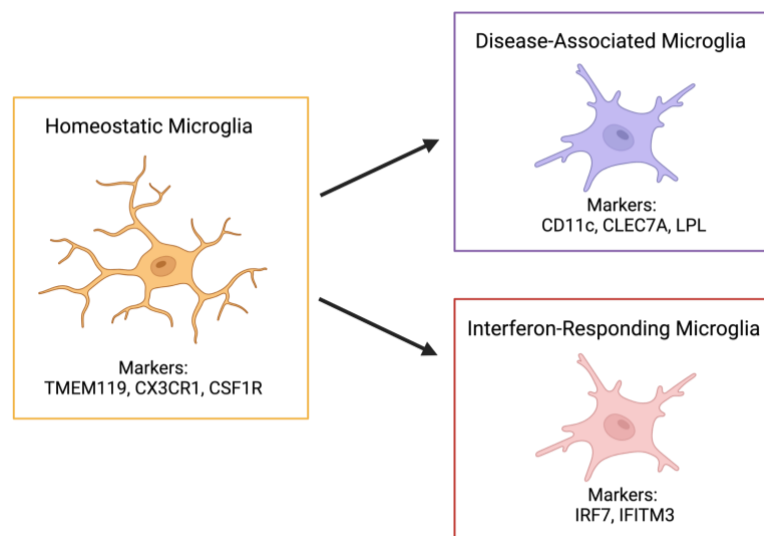


Figure 5.2: Markers of microglia states that can be leveraged for conditional manipulation. Microglia gene/protein identifiers that can be used to develop conditional knock-out models for in vivo studies of specific microglia states in AD progression.

5.4 Evaluation of neural activity to reflect age- and AD-related synaptic structural adaptations across mouse strains

Throughout this work I hypothesized that AD resilience was caused by cell types that drive A β -induced synaptic damage, but it is feasible that there are genetic factors promoting neurons to be protected from neuropathology. This is supported by a recent study of a patient carrying autosomal dominant AD mutations but exhibiting cognitive resilience³⁵⁸. In this case report, a patient who was a carrier of the *PSEN1*-E280A mutation (which confers high risk to developing fAD) remained cognitively intact until the age of 67 before exhibiting dementia symptoms. This patient also carried high amounts of A β and Tau pathological burden which was discovered upon histological and imaging analyses of their brain, making researchers curious as to why they failed to exhibit cognitive symptoms. Interrogative genetic studies of this individual identified a putative protective variant in the *RELN* gene, which encodes a protein with critical importance to neuronal development. During early development and postnatal stages, the Reelin protein is responsible for proper migration of cortical neurons in order to properly position to develop critical neural circuits for brain function³⁶⁹. In the adult brain, Reelin functions at the cortical neuronal pre-synapse, binding to apolipoprotein receptor 2 (ApoER2) and very-low-density lipoprotein receptor (VLDLR) to initiate Src-family tyrosine kinase (SFK) phosphorylation of the NR2 subunit of the N-methyl-D-aspartate (NMDA) receptor, resulting in potentiation and Ca²⁺ influx and neurotransmitter release³⁶⁹. While continued functional studies are still necessary to fully test the impact of *RELN* variants on disease resilience, this nominated possible pathways that could drive AD neuronal resilience through synaptic signaling. It is possible that neurons from PWK mice, and a similar manner to this human patient, upregulate or alter neurodevelopmental signaling pathways (such as ApoER2 and VLDLR receptor

pathways that initiate DAB1 downstream signaling to initiate neurogenesis and brain injury repair³⁷⁰) to protect neurons from A β -induced damage. Studies to test this further will implore single cell transcriptomics to provide evidence for the cell-types and pathways that differentiate to PWK resilience over B6, and in a more unsupervised and high-throughput manner. This would then identify molecular targets (possibly *RELN*) and pathways (neuronal development, ApoER2 signaling, or neuroprotection signals), that can be targeted to promote neuronal resilience.

An additional attribute to PWK resistance/resilience are the age-related structural changes made to dendritic spines (as observed in **Chapter 3**). These modifications suggest PWK synapses decreased electrical filtering of the signal at the spine to the parent dendrite, allowing for increase neuronal excitability with age. Since spine synaptic structure is a reliable measure of functional properties of the neuron^{92,155,157,159,160}, this suggests there are intrinsic mechanisms within PWK mice that drive these neuron-centric changes during aging, as well as their resilience to AD. I predict these structural synaptic changes could be a key to understanding the resistance/resilience of age- and AD-related synaptic (and possibly cognitive) decline. However, to test this prediction, follow-up studies in PWK and other mouse strains are required, particularly focused on *in vivo* neuronal excitability and behavior. Commonly used approaches for understanding *in vivo* functional activity are assays that can measure cellular calcium (Ca²⁺) dynamics³⁷¹. Since Ca²⁺ is a common cellular messenger, is often mobilized across networks upon evoked neural activity. Ca²⁺ imaging techniques require the neurons to express a calcium indicator, such as GCaMP6, which is a genetically encoded Ca²⁺ sensor and synthetic fusion of GFP and calmodulin (CaM) protein. When Ca²⁺ binds, it activates the encoded GFP, causing the neurons expressing GCaMP to fluoresce, which can be visualized using multiphoton imaging or fiber photometry. These properties measure neural activity in an awake, freely-moving mouse, which allows for measuring

functional changes in neural circuits while performing different behavioral assays^{372,373}.

Furthermore, longitudinal imaging of neural circuits under various experimental conditions including aging can also be performed using these approaches, as specific neurons can be targeted with a virus to express GCaMP expression. Therefore, the neural circuits analyzed in this thesis that were analyzed for structural dendritic spine changes (in PFC and hippocampal CA1), then related to longitudinal changes in neural activity and performance on behavioral assays³⁷⁴.

While the findings in **Chapters 3 & 4** propose structural adaptations that neurons in PWK mice use to exhibit resistance/resilience to synaptic loss and cognitive decline, there is interest to connect these structural changes back to functional consequences. By leveraging *in vivo* imaging approaches, such as Ca^{2+} imaging, I would be able to test the hypothesis that B6 lose neural excitability with aging while PWK either remain intact or adapt their circuits to increase neural activity. Conversely, I could also test the resilience of PWK.*APP/PS1* hippocampal neurons to $\text{A}\beta$ pathology, and consequentially identify the functional changes that occur in the hippocampus from B6.*APP/PS1* mice. I could also evaluate how excitatory neural activity increases during behavioral assays (such as the delayed spatial assay), and how it is dampened (or fails to dampen) in animals susceptible (or resistant) to age-related cognitive deficits. Importantly, *in vivo* functional measurements can longitudinally assess loss of neural activity in the same mouse, adding more power to these studies. By synthesizing three data types proposed here (neuronal single cell transcriptomics, *in vivo* activity, higher-order behavior), I could identify genes and pathways within neurons that relate back to PWK cognitive resistance/resilience, as well as mine human data to identify if these pathways differentiate resilient from susceptible individuals. These neuron-centric studies would provide a more wholistic viewpoint of the cellular and molecular events that mediate synaptic stability to aging and AD.

5.5 Contribution of astrocytes to aging resistance and AD resilience

In addition to microglia, microglia states, and neurons, another cell-type that could be contributing to resilience mechanisms that was not investigated here are astrocytes. Astrocytes, another major glia cell of the nervous system, have demonstrated importance as a support cell and driving neuropathology during neurodegeneration. Being a cell type that outnumbers neurons by more than five-fold, they tile the entire CNS to provide critical functional and structural support, particularly with maintaining neuronal synapses³⁷⁵. It was recently discovered that astrocytes preferably prune excitatory over inhibitory synapses³⁵⁸, further suggesting they could have a more critical role on CA1 pyramidal cells examined throughout this thesis.

Similar to microglia, astrocytes become reactive and have been found to develop functional states when exposed to disease-related pathologies^{235,376}. Since the presence of variable astrocyte states has yet to be explored across wild-derived *APP/PS1* mice and related back to known disease resilience/susceptibility, it is possible that there is an existence (or lack) of an astrocyte state in PWK that are protective to CA1 synapses. If a particular state didn't show overt involvement, it is also possible that PWK astrocytes adapt molecular pathways that make them efficiently respond to disease pathologies and protect neuronal synapses. This links back to the results in **Chapter 2**, in which m7 (enriched for lipid biosynthesis pathways) was a critical regulator of AD-BXD cognitive outcomes (**Figure 2.6**). Genes in this module were equally enriched in microglia and astrocytes (**Figure 2.2**), suggesting that lipid biosynthesis pathways in these cell types may be the critical mediators of disease resilience. As I didn't specifically investigate pathways in these cell types in this thesis, a more thorough investigation could be warranted, being that lipid handling has become a pathway of increased importance in AD due to the implications of APOE genetic risk in LOAD³⁷⁷. Future experiments that

evaluate astrocyte and microglia lipid dynamics across diverse genetic contexts might critically link the dysregulation of these cell-types or how they promote AD resilience.

5.6 Alternative AD mouse models

In **Chapter 1** I discussed the traditional use of transgenic mouse models in AD research, and the many strengths and weaknesses of these systems. To address the limitations imposed by transgenic models of AD (i.e., reliance on a promoter and artificial transgene expression discussed in **Chapter 1**), novel knock-in mouse models have been developed that express disease-causing fAD mutations without the reliance on the overexpression of an artificial transgene. One example of this is the *App*^{SAA} that was first engineered to convert the B6 mouse *App* locus to contain an identical sequence to the human *APP*, by substituting three amino acids (APP G676R, APP F681Y and APP 684H) in the A β ₁₋₄₂ region. This was followed by introducing fAD-linked mutations into the humanized locus: the K670N/M671L **Swedish**, E683G **Arctic** and T714I **Austrian**³⁵⁷. Initial studies suggest that homozygous *App*^{SAA} mice recapitulate pathological features of AD including the accumulation of parenchymal A β plaques and microglial activation³⁵⁷. With the development of this model, and its open access to the scientific community, future studies can be conducted that leverage the natural mouse expression of these amyloidogenic genes, rather than overexpression of an artificial transgene. These mice are currently generated to be congenic on the WSB, CAST, and PWK genetic backgrounds, allowing for further investigations of genetic context on fAD-related phenotypes such as cognition, neuropathology, and synaptic health. Since pathological A β accumulation is not as aggressive as transgenic fAD models, and age is the primary risk factor for AD, these new wild-derived *App*^{SAA} can be aged for longer periods so that age x A β interactions can be studied in how they are impacted by genetic context. This

would complement my current work studying early factors of AD, and allow for additional predictive analyses of how genetic context drives disease trajectories.

Outside of fAD there are recent mouse models that incorporate mutations found in LOAD that could be used in future studies. The Model Organism Development and Evaluation for Late-Onset Alzheimer's Disease (MODEL-AD) consortium was established in 2015 with the goal of creating more translatable mouse models of LOAD, and make them publicly available to researchers worldwide³⁷⁸. While more than 40 mouse models have been developed to date, several have been characterized in greater detail to provide evidence that the established strains develop similar molecular, cellular, and neuropathological profiles to those of LOAD patients. The first iteration of these models, LOAD1, is a double-mutant strain that carries a humanized *APOE*^{ε4/ε4} knock-in allele and *Trem2* R47H point mutation, which have both been identified as high genetic risk factors for developing LOAD in human populations^{31,379}. These mice display important molecular and serum biomarker characteristics that can be studied to better understand how these variants cause increased LOAD risk. These models are currently being built on to incorporate additional AD risk, such as humanized Aβ, to further enhance the face validity of translational mouse models to display AD phenotypes. While LOAD models are not currently available on genetic backgrounds outside of B6, future work can create congenic models of the LOAD variants that are hypothesized to be the most impactful at driving AD susceptibility in that model. For example, *Abca7* is a gene expressed in microglia and astrocytes, and one of the genes carrying a LOAD-relevant mutation in these new mouse models. Since my hypothesis for this work was that microglia or astrocytes are driving disease susceptibility, future work could study how the introduction of LOAD-relevant mutations in *Abca7* may create susceptibility to CA1 synaptic loss, and further evaluate if this appears to be driven by microglia, astrocytes,

or a combination thereof. While *Abca7* was presented as an example based on the cell-types it is known to be expressed in, additional data such as snRNAseq proposed in the section previously will nominate additional, LOAD-relevant, gene candidates that can be interrogated using the MODEL-AD LOAD models in the future.

5.7 *In vitro* approaches to study AD cell type-specific resilience mechanisms

Cell culture systems provide a level of control that is often not afforded by the complexity in mouse models, such as the isolation of a cell type, environmental exposures, and high-throughput experimentation. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have become popularized due to the ability to study disease mechanisms in both mouse- and human-derived cell lines³⁸⁰. With recent establishment of differentiation protocols for induced microglia, astrocytes, and neurons (among other cell types), the cellular consequences of AD-linked mutations have become better understood³⁸¹. The strength of cell culture models is the environmental control of cellular exposures, and the simplified systems of being able to understand how structured manipulations (such as genetic variation/variants) alters cellular function. For example, assays that measure pro-inflammatory cytokine secretion, phagocytosis of synaptosomes, and migration measures can provide a thorough functional picture of differences between microglia cell lines derived from multiple genetic contexts that may not be as accessible *in vivo*. Additionally, assays measuring calcium mobilization or electrical array recordings have been developed to provide information about astrocytic and neuronal cultures³⁸². Advancements in mixed culture systems, 3D scaffolds, and organoids also evaluate how cells interact with each other in a controlled manner, and recapitulate the complexity of the CNS^{383–385}.

One approach by which cell type-specific mechanisms of PWK resistance/resilience could be investigated with much more control is through the derivation, differentiation,

and functional analysis of ESCs from each of these strains. As derivation protocols for ESCs from mice have dramatically improved³⁸⁶, it is now possible to readily generate pluripotent lines from diverse mouse strains. By evaluating how differentiated cells from each of these mouse strains perform on assays, I would be able to gather information about how functional differences in these cells relate back to the more complex structural information. For example, differentiated microglia from B6 could be secreting different levels of pro-inflammatory cytokines (IL-6, TNF, IL-1 β) when exposed to A β when compared to microglia from PWK, which could indicate why there are differences in the neuronal response to microglia *in vivo*. Furthermore, multielectrode array measurements of differentiated neurons could provide dynamic information for how B6 versus PWK neurons respond to A β , co-cultured astrocytes, co-cultured microglia, or a combination thereof.

To offer translatability of findings in mice, and one of the strengths of mouse ESCs, is the ability to evaluate how cultured mouse cells relate to differentiated cells from human iPSCs. While models derived from human iPSCs have often been performed on lines from a single individual, a recent study generated induced neuronal cultures of iPSCs from 53 individuals in the ROSMAP cohort. These results showed that specific A β and tau species produced by neurons corresponded to postmortem pathological brain attributes from the same individuals, as well as polygenic LOAD risk³⁸⁷. Furthermore *in vivo* transcriptional signatures exhibited a strong concordance with brain bulk RNAseq signatures, providing support for the utility of these model systems. These findings act as a translational approach for mouse systems, by identifying individuals that are cognitively susceptible and resilient, and comparing their functional cellular measures from differentiated iPSCs to mouse ESC cultures from B6 and PWK strains. Furthermore, since human iPSCs were derived from individuals in the ROSMAP study, I

could relate findings from those individual cell lines back to patient characteristics such as postmortem brain RNAseq and neuropathology.

There are caveats to the validity of cell culture models to study complex human disorders, a major one being that 2D cultures of human iPSC-derived microglia do not recapitulate RNAseq signatures found in purified human brain microglia^{388–390}. One way to combat this is to expose cultured microglia to stimuli they often encounter in the brain (synaptosomes, myelin debris, synthetic A β fibrils), which has been found to recapitulate transcriptomic attributes of *in vivo* brain microglia³⁹¹. To address additional limitations of culture systems, chimeric mouse models have been generated in which iPSCs differentiated to a particular CNS cell type are xenotransplanted into immunodeficient mice^{390,392}. This has been most effectively demonstrated using human-derived microglia, as xenotransplants show greater transcriptomic conservation to isolated human microglia, as well as functional responses to focal injury, neuronal phagocytosis and inflammatory challenge³⁹⁰. Transplanted microglia show a robust cytokine-mediated inflammatory response to injected oligomeric A β , making further study of microglia using chimeric mouse models more relevant to evaluating contributions to AD pathology³⁹². New mouse host systems such as the *Csf1r*^{FIRE/FIRE} have been developed in which resident macrophages are selectively depleted, allowing for the transplantation of iPSC/ESC-derived microglia³⁹³. These mice have been crossed with the 5XFAD mouse model to provide A β pathology in chimeric models, creating higher face validity towards AD research³³⁰. One thing to note is these mice are on a CBA/CaJ genetic background, and additional crosses to B6 and PWK would be necessary to make them congenic.

The concept of human-mouse chimeras is an important factor to be expanded upon in future work, especially when disentangling the intrinsic versus extrinsic cellular mechanisms that promote AD susceptibility and resilience. One experiment would be a

microglial swap, where *Csf1r*^{FIRE/FIRE}-5XFAD (or *APP/PS1*) mice (on the B6 or PWK background) are transplanted with ESC-derived microglia from B6 or PWK mice. I could evaluate how PWK microglia respond to the B6 host brain microenvironment (or the reverse), to establish if the host environment versus the transplanted microglia as more important for driving disease phenotypes such as synaptic loss and cognitive decline. I could also evaluate the transcriptomic states of these microglia after transplantation to determine if the host environment drives their development or if they are more intrinsically dictated by genetic context. Comparing functional outcomes of ESC-derived mouse microglia to iPSC-derived human microglia from resilient and susceptible individuals will provide key insights into how gene background influences cell types in the CNS during AD progression.

5.8 Mapping genetic factors driving resilience using novel mouse populations

The power of genetically diverse mouse systems like the ones described in this thesis is the ability to harness their genomes to identify candidate variants and genes driving higher-order traits. This was one of the general concepts behind the creation of the BXD GRP (discussed in **Chapters 1 & 2**), where B6 and D2 mouse strains were known to exhibit differences on a wide array of traits that could be mapped by to genetic differences between the two strains⁴⁶. However, B6 and D2 are two inbred strains that only diverge by 5 million SNPs⁵¹. This limited diversity creates large haplotype blocks in the genome that cannot be resolved through traditional genetic mapping approaches. To address the limitations of traditional recombinant inbred panels, the DO and CC populations were developed, which incorporated additional parental strains including wild-derived WSB, CAST and PWK that diverged from traditional laboratory inbred strains by over 20 million SNPs^{49–51}. Mice from these panels exhibit much greater trait diversity, as well as carry smaller haplotype blocks which makes identifying genetic

candidates that regulate these traits through mapping approaches much more precise. GRPs such as the BXD, DO and CC populations have provided valuable insight into the genetic mechanisms that regulate higher order disease traits, gene expression, and protein abundance (among others), but they are limited in accessibility by the number of animals required to perform these studies, as hundreds of animals are required to be statistically powered to observe variability in a given complex phenotype³⁹⁴. There are additional, more traditional, mapping approaches available that can be leveraged to resolve genetic modifiers of a particular trait.

One of the first steps in a genetic mapping experiment is to evaluate how the parental lines perform on a particular trait. Results from this thesis have provided such evidence, with PWK and B6 displaying opposing effects on cognitive performance and synaptic loss due to aging and A β pathology. Since future experiments have been proposed throughout this section to dissect the mechanisms that drive this divergence, such as single cell transcriptomics and proteomics, there are also genetic mapping approaches that could leverage this divergence between B6 and PWK such as the generation of an F2 intercross. This is similar to traditional studies between B6 and D2 that were foundational for the development of the BXDs^{395,396}, a B6 x PWK F2 intercross (BxP) panel can be generated to identify genetic factors that create PWK resilience. Using the delayed spatial assay, BxP mice can be screened for cognitive, neuronal, and synaptic attributes, which can be genetically mapped to a relatively high resolution due to the high degree of genetic diversity (>20 million SNPs) that exist between the two strains. Additionally, individual mice with extreme divergence can be chosen for more detailed synaptic analyses to determine how their genetic background is modifying their dendritic spine stability. After performing traditional genetic mapping, genetic candidates can then be manipulated in vivo to more specifically evaluate how they promote or impair resilience phenotypes in the B6 and PWK founder strains. These data can be

synthesized with additional single cell omics to further nominate a cell-type and pathways that are involved in PWK resilience.

5.9 Conclusions and future directions

As described throughout this discussion, there are many future avenues that can be taken to functionally and mechanistically dissect the genetic attributes that promote AD resilience. This can be done through high-throughput analyses of protein abundance, lipid and metabolomic profiles to see how they correspond to brain transcript abundance, as well as an analysis of AD and aging molecular pathways at a single cell resolution. This provides a more personalized approach to AD treatment, as the disease is heterogenous in nature, so understanding the factors that drive individuals to be resilient will help with the development of therapeutics to promote resilience attributes in patients that are not naturally protected. A more detailed assessment of the cell-types outside of microglia that contribute to AD resilience is warranted, which can be done using conditional genetic manipulations of mouse models that are readily available. I have also proposed additional mechanistic and functional studies to determine how the structural adaptations of neurons that I observed in mice relate to *in vivo* function. An important consideration when using model organisms of a human condition is the translational aspect of our findings. Being that AD resilience and cognitive aging resistance is well-reported, and identified as a heritable attribute^{25,331}, our next steps in the identification of factors that are promoting PWK mouse resilience would be to mine human studies for similar genetic factors which can be readily translated. These genetic factors would be grouped into pathways, in which proteomic targets can be identified for future study to delay AD progression or promote cognitive longevity during early disease stages, where much of the damage to the CNS can be reconciled.

Each chapter provides valuable findings highlighting the strong contribution of genetic context in complex human diseases, showing how the neural landscape is altered by the interaction of genetic context and age-related insults. While there is much more to be investigated using the novel PWK mouse strain, the data presented here are important in providing additional evidence that genetic context determines how cells in the CNS respond during aging and AD, paving the way towards precision approaches that are more personalized rather than generalized to a complex population.

Chapter 6: Bibliography

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