Behavioral Profiling of 5xFAD Mice: Investigating β-Amyloid–Driven Deficits in Cognitive and Non-Cognitive Domains

Nilufar Imomdodova
Department of Neuroscience & Psychology
Lake Forest College
Lake Forest, Illinois 60045

As the quality of life improves and human lifespan extends, the prevalence of Alzheimer's disease (AD) continuously grows. AD is a devastating neurological condition affecting millions and driving extensive research to uncover its mechanisms. Because amyloid-β plaque deposition is the earliest pathological hallmark of AD, therapies aimed at slowing plaque formation are most effective when initiated at the very onset of disease—making sensitive, early behavioral biomarkers imperative.

The 5xFAD mouse, which develops aggressive amyloid pathology, offers a powerful platform for uncovering early behavioral signatures. Here, I subjected 5xFAD and wild- type littermates to a comprehensive battery of assays spanning decision-making, attention, exploration, memory, and other non-cognitive domains. We hypothesized that plaque-driven behavioral deficits would surface as soon as plaques emerged.

Although robust amyloid deposition was detected in the subiculum, its burden correlated only weakly and non-significantly with behavioral performance. These results underscore the need for larger sample sizes, plaque quantification in additional regions (e.g., cortex), and further refinements to assay sensitivity in order to pinpoint reliable behavioral indicators of early AD.

I. Introduction

Where did I leave my keys? Did I turn off the iron before I shut the door? Did I lock the door? Forgetting is a universal experience—something that happens to all of us, often daily. It usually brings a touch of irritation or frustration as we struggle to recall specific details and replay the particular moments in our minds.

Now imagine waking up day after day with the same sense of frustration and inability to remember even the most basic things. You first forget where you usually keep your toothpaste. Then you can't recall where you live, and eventually, you no longer recognize your loved ones.Scary, isn't it?

This is what daily life can feel like for a person with Alzheimer's disease. It is not just their memory that fades, but their very self—their identity, their life, and everything that once defined them—all slipping away.

1. Overview of Alzheimer's Disease

1.1 Alzheimer's Disease Facts and Figures

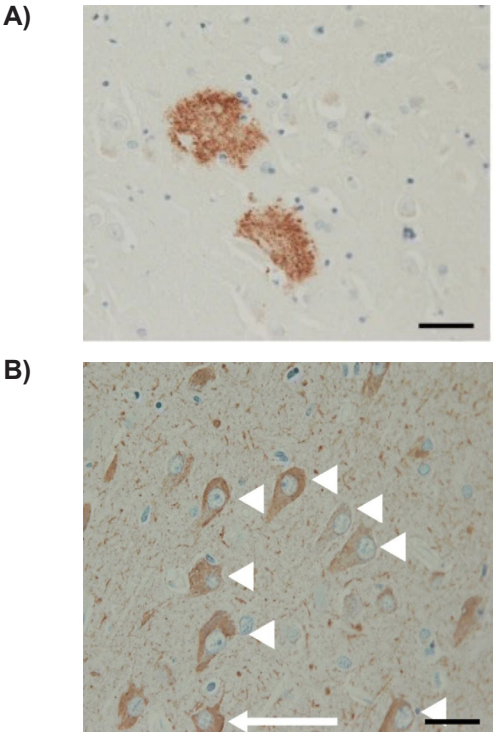
Alzheimer's disease (AD) is a neurodegenerative disease and the most common type of dementia, making up 60-70% of the cases (WHO, 2023). According to the Alzheimer's Association, there are approximately 6.9 million people living with AD in the USA and the numbers are expected to grow to 13.8 million by 2050. Age is the number one risk factor for developing AD, which explains the continually increasing prevalence of the disease as lifespans expand globally (Alzheimer's Disease Facts and Figures, 2024). The number of deaths due to AD was approximately 119,399 in 2021 worldwide (Alzheimer's Disease Facts and Figures, 2024). AD is not just a devastating condition for the patients and their families, but also a significant burden to the country's economy. In 2020, Americans spent 196 billion US dollars in direct medical costs and caregiver time equivalent to 254 billion dollars (Nandi et al., 2024). The National Institutes of Health (NIH) approved an additional fund of 100 million dollars aimed at AD research, making a total of 3.4 billion dollars in 2024 (Alzheimer's Association, 2024).

1.2 AD Pathology, Etiology, Symptoms, and Risk Factors

The two molecular hallmarks of AD are beta-amyloid (Aβ) plaques and tau tangles (Fig. 1A & B) that precede and cause physiological deteriorations to the brain. Such changes are atrophy defined by tissue and brain volume loss (enlarged ventricles and shrunken gyri) (Fig. 1C). Also, neuronal death and microglial inflammation in the hippocampus correlate with the progressive decline of cognition (Rao et al., 2022). There are two subtypes of AD: Late Onset (LOAD) and Early Onset (EOAD). The overwhelming majority of the patients (95%) have LOAD (after 65 years old), which is sporadic. The rest (5%) is EOAD (before 65 years old), which is determined by genetic predisposition and displays more aggressive progression (Mendez, 2019). Along with the pathological deterioration, there are also several stages of symptoms that AD patients go through as the disease progresses. The stages include preclinical (development of plaques and tau tangles with no pronounced behavioral deterioration), mild (repeating questions, memory loss, poor judgment), moderate (withdrawal from social activities, shortened attention span), and severe (inability to communicate, weight loss, loss of bladder and bowel control) (National Institute on Aging, n.d.).

In terms of Aβ plaque accumulation, a thorough plaque localization analysis showed that first deposits are seen in the neocortex, then spread into limbic regions including the hippocampus and entorhinal cortex, as well as the basal ganglia, and ultimately accumulate in the cerebellum. On the other hand, Braak and Braak showed that neurofibrillary tangles initially aggregate in the lower brainstem (locus coeruleus) and then spread into the hippocampus, eventually spreading into neocortical areas (Hampel et al., 2021) (Fig. 2).

Autosomal dominant mutations in genes such as APP, PSEN1, and PSEN2 that alter Aβ precursor protein are related to the EOAD, whereas age-related changes and sporadic mutations that are more difficult to identify underlie LOAD (Uddin et al., 2021). Some of the LOAD related risk factors include mutations in APOE (APOE 4 isoform), BIN1, CLU, PTK2B, CR1, MS4A2, PICALM, IQCK, and TREM2 (Chen, Petty, Sha, et al., 20).



*This author wrote this paper as a senior thesis under the direction of Dr. Eun Jung Hwang.

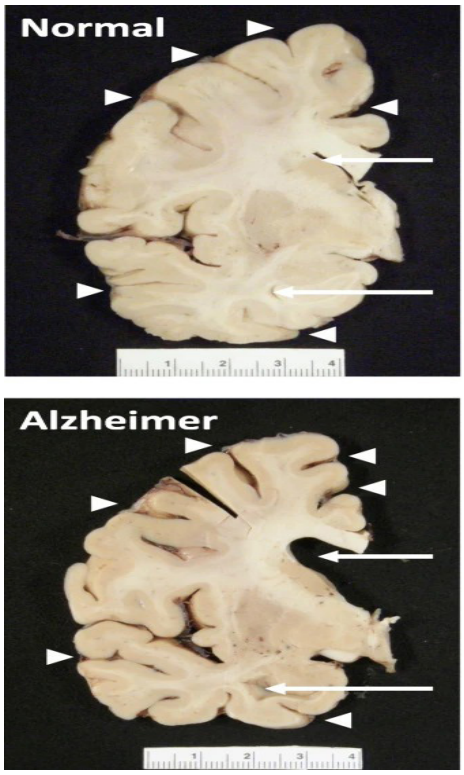


Fig. 1. **Micro- and Gross-level Pathological Changes in AD patients**. (**A**) Densely packed neuritic Aβ plaques in AD patient brain. (**B**) Matured neurofibrillary tangles (denoted by arrows) and pre-tangles (denoted by arrowheads). (**C**) Atrophy of the brain. On top is the normal/healthy brain section. On the bottom, AD brain shows marked atrophy, dilation of the lateral ventricle (top arrow), and a shrunken hippocampus (bottom arrow) and shrunken gyri (arrowheads). (DeTure & Dickson, 2019).

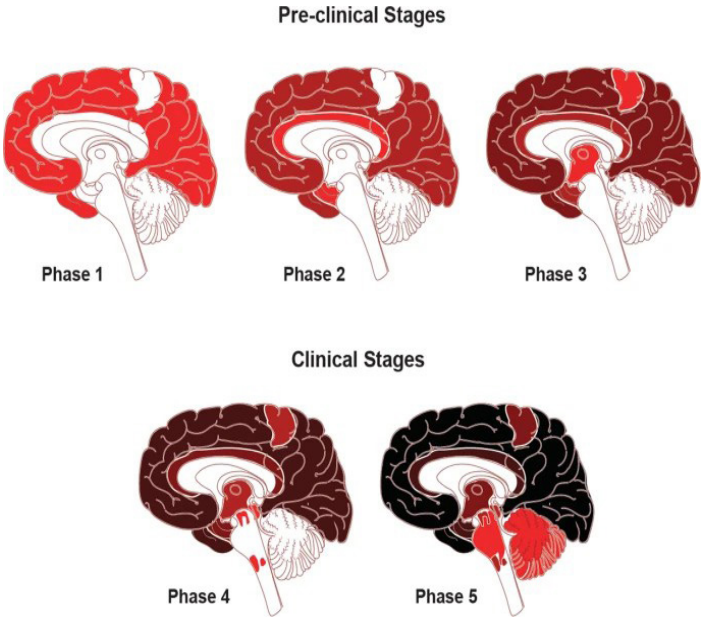


Fig. 2. **Amyloid Plaque Accumulation in Different Stages of AD.** Initial aggregations are depicted in bright red, with continued deposition in the same region represented by progressively darker shades, illustrating the progression from stage 1 to stage 5. Plaque deposition begins in the neocortex (stage 1) gradually spreading to limbic system (stage 2), thalamus (stage 3), pons (stage 4), eventually reaching the cerebellum in stage 5 of the disease. (Hampel et al., 2021).

2. Disease Process of AD

2.1 Aβ Plaques Formation

The Aβ peptide plays a crucial role in AD pathology and is formed because of altered cleavage of Amyloid Precursor Protein (APP) by β and γ secretases. APP is located on chromosome 21 and belongs to the family of proteins that also includes APP-like proteins 1 (APL1) and 2 (APL2), all of which are type-I transmembrane proteins in mammals (Coulson et al., 2000; Wasco et al., 1992). The role of APP is still being explored, but most studies suggest that it may be responsible for neurite outgrowth (Bibel et al., 2004), synaptogenesis (Moya et al., 1994), cell adhesion (Soba et al.,2005; Wang et al., 2009), and other functions substantial for cell activity and proliferation (Zhang et al., 2011).

APP is found in several isoforms produced by alternative splicing of the exons (coding region of gene) 7, 8, and 15 (Menéndez-González et al., 2005). The most abundant form of APP in the brain is APP 695 — produced mainly by neurons and lacks Kunitz-type serine protease inhibitory (KPI) domain sequence (KPI-). Whereas APP 751 and 771 are predominantly expressed in glial cells and contain KPI (KPI+) sequence (Chen et al., 2017). The concentration of KPI+ APP mRNA significantly increases, whereas KPI- APP mRNA decreases in AD brain. KPI+ APP mRNA level is also positively related to Aβ production and accumulation (Menéndez-González et al., 2005). Considering the differences between the KPI- and KPI+ APP isoforms concentrations, it is crucial to understand the underlying molecular mechanism of the KPI. KPI is a ubiquitous protein that belongs to the protease inhibitor I2 family and is found across different species. Structurally, it is a relatively small 60-80 amino acid residue-protein (de Magalhães et al., 2018). KPI containing APP isoform has been shown to be prone to homodimerization with more molecular mechanisms to be discovered (Ben Khalifa et al., 2012; Byun et al., 2023). Evidence also suggests that KPI+ APP interacts with α-secretase, inhibiting its normal function. This inhibition might be key to a shift toward β-secretase cleavage, increasing Aβ production and contributing to AD pathology (Lesné et al., 2005).

APP is synthesized in the endoplasmic reticulum and transported to the trans-Golgi network (TGN), where it undergoes sorting and modification. From the TGN, APP is directed to the plasma membrane, early endosomes, and other intracellular compartments. Its localization and trafficking are dynamic, with endosomes being a key site for its amyloidogenic processing into amyloid-beta peptides, which are implicated in AD pathogenesis (Jiang et al., 2014). Following prior cleavage by β-secretase, processing of APP by γ-secretase generates Aβ peptides of varying lengths.

Another key gene implicated in AD is PSEN1 that is known to be the most common genetic cause accounting for ~6% of EOAD cases. PSEN1 encodes presenilin-1 (PS1), which functions as the catalytic subunit of γ-secretase, an intramembranous protease that cleaves a variety of type 1 transmembrane proteins, most notably APP (Kelleher & Shen, 2017). Most PSEN1 mutations are heterozygous showing autosomal dominance heritance pattern. Two specific mutations, Asp40del (delGAC) and Ala79Val, show a stronger link to EOAD, compared to others. Both mutations lead to high production of Aβ42, but not Aβ40. In contrast, mutations such as Gly209Arg, Gly209Val, Leu235Pro, Cys410Tyr, Leu435Phe lead to decrease in Aβ40 production. Current data shows that loss of function in PS1 is the driving force of AD progression. Although not fully established, some evidence suggests that PSEN activity may result in abnormalities in synaptic functions, leading to neuronal loss, and tau hyperphosphorylation (Bagaria et al., 2022).

In a healthy brain, APP is typically cleaved by α secretase that maintains its normal structure and function of APP. This cleavage produces sAPPα and C83. sAPPα is crucial for neuroplasticity and cell survival (Furukawa et al., 1996) and is protective against neurotoxicity (Han et al., 2005). sAPPα has also been positively linked with activation of muscarinic acetylcholine receptors (Haass et al., 1995). C83 is subsequently cleaved by γ secretase, forming p3 and APP intracellular domain (AICD) (Fig. 3). According to

Kuhn et al (2020), there is no conclusive data on the role of the p3 peptide. Some researchers claim its neuroprotective role, while others highlight its neurotoxicity. Similarly, in AD brain, APP cleavage by β secretase produces sAPPβ and C99. C99 is further cleaved by γ secretase, producing Aβ and AICD. Considering that AICD is produced in both cases, there is no solid understanding of its contribution to the development of AD. Whether it is toxic to the brain or protective is an important topic of debate (Muller et al., 2008) (Fig. 3). Most relevant to AD are the two isoforms of Aβ, Aβ40 and Aβ42, which are both found in AD plaques. Those isoforms can form three types of aggregates: Aβ40, Aβ42, and Aβ42/Aβ40. Compared to Aβ42, Aβ40 takes a much longer time to aggregate, and is therefore less likely to form insoluble deposits or drive pathological changes (McGowan, 2005). The third type of aggregates, Aβ42/Aβ40, displays a delay in accumulation similar to Aβ40. Only Aβ42 formation is a specific isoform that is specifically implicated in AD pathology and neurotoxicity (Gu & Guo, 2013; Kuperstein et al., 2010). Structurally, through nucleation, Aβ monomers aggregate into different types of assemblies: oligomers, protofibrils and amyloid fibrils. Aβ fibrils are larger and insoluble and are capable of forming amyloid plaques, whereas oligomers are soluble and can spread around the brain (Chen et al., 2017). The latter aggregates at the early stages of the disease and are considered to be highly toxic, driving the amyloidogenic pathway in AD (Sehar et al., 2022). Aggregated Aβ plaques can exist in both loosely and densely packed forms. Research indicates that densely packed plaques do not form spontaneously but rather through a process involving microglia. Microglia organizes the loosely packed Aβ oligomer structures into dense-core plaques, thereby potentially limiting their toxicity. In other words, microglia-mediated plaque compaction may reduce the harmful effects of Aβ accumulation on surrounding neurons (Huang et al., 2021).

2.2 Neurofibrillary Tangle Formation

Tau protein is encoded by MAPT gene located on chromosome 17. Exons 2, 3, and 10 are crucial for canonical functioning of tau. Exons 2 and 3 are translated to N1 and N2 aspects of N- terminal responsible for signal transduction and membrane interaction. Exon 10 on the other hand encodes the R2 region—the second repeat in the C-terminal microtubule-binding domain and is strongly associated with neurodegeneration. Alternative splicing of exon 10 results in either 3-repeat (3R) or 4-repeat (4R) tau isoforms, depending on whether the exon is excluded or included. Since the microtubule-binding domain of tau mediates its interaction with microtubules, it plays a key role in maintaining microtubule stability, regulating their dynamics, and supporting axonal transport (Park et al., 2016). The boundary between exon 10 and intron 10 contains an RNA sequence that forms a stem-loop structure through self-complementary base pairing. This stem-loop is a hot spot for *MAPT* gene mutations, as numerous pathogenic intronic variants are concentrated within this region. These mutations destabilize the stem- loop, leading to aberrant splicing of exon 10 by increasing its inclusion. The resulting alteration in RNA secondary structure enhances spliceosome accessibility, thereby promoting mis-splicing (Kar et al., 2011; McCarthy et al., 2015). In a healthy brain, the levels of 4-repeat (4R) and 3-repeat (3R) tau isoforms are typically balanced at an approximate 1:1 ratio. Disruption of this equilibrium has been implicated in the pathogenesis of various tauopathies. While no definitive association has been established between a specific tau isoform and AD, emerging evidence suggests that distinct subtypes of AD-related tauopathies may exist, potentially influenced by alterations in tau isoform expression or ratio (Liu & Gong, 2008).

Considering the role of tau in maintaining the stability and architecture of microtubules and subsequently axons, tau may play a crucial role in signal transduction and viability of the neuron (Wang & Liu, 2008). The accumulation of the tau protein starts at the brainstem and limbic systems, progressively spreading through the entire brain (Gabitto et al., 2024). Tau protein goes through multiple post-transcriptional modifications such as glycolysis, nitration, truncation, etc., however, phosphorylation is the most well-studied modification.

Phosphorylation is one of the most common causes of tau dysfunction which leads to tauopathies in AD (Avila et al., 2004). The role of Aβ plaques in tau tangle formation is undeniably important as Aβ plaques directly promote tau phosphorylation and thus its tangle formation. Other than phosphorylation, cleavage of tau can also contribute to tangle formation. Caspases - a family of 14 enzymes, cleave substrates at specific aspartic acid (Asp) residues.

Cleavage by caspase at tau's C-terminus or N-terminus leads to impairment in mitochondrial bioenergetics, weakening of axonal transportation, neuronal injury and cognitive decline.

Caspase-3 cleaves tau at the C-terminus tail at Asp421, which removes 20 amino acids, promoting rapid assembly of neurofibrillary filaments. This cleavage event contributes to neurite loss, mitochondrial fragmentation, and tangle aggregation, driving cognitive decline (Rizzi & Grinberg, 2024). Aβ can indirectly trigger caspase 3 (CASP3) to produce a self- aggregating and neurotoxic tau oligomer. (Zhang et al., 2023). More specifically, Aβ induces mitochondrial dysfunction, which leads to the release of cytochrome-c (a molecule released by mitochondria when cell receives apoptotic signal) and caspase-9 activation, activating downstream CASP3.

Although less studied, caspase-6 also plays role in AD pathology. The levels of caspase-6 were negatively related to cognition and the 14-441 fragment of Asp13 cleaved by this enzyme plays role in tangle maturation. Unlike caspase-3, its activation seems to be independent from Aβ and starts at an earlier stage of AD. Taken together, tau tangle formation is a complex process that involves several independent mechanisms and can vary depending on the stage of the disease. It can also be Aβ dependent, posing an important question about the interplay of those AD biomarkers.

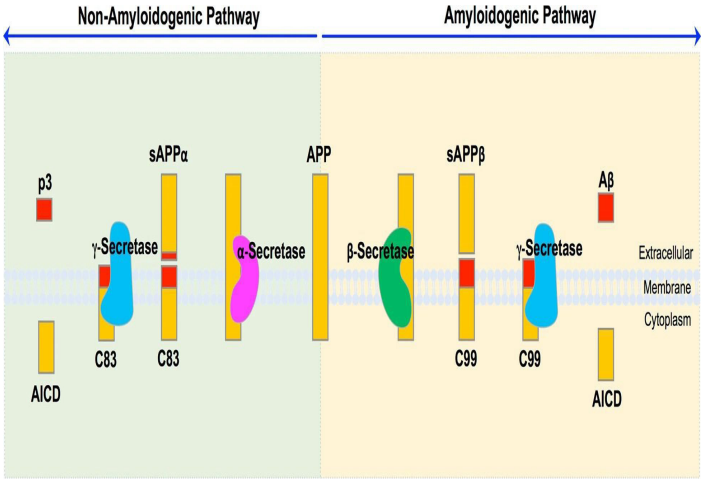


Fig. 3. **APP Healthy and Pathological Cleavage Mechanisms.** The amyloidogenic pathway involves β-secretase and γ-secretase, while the non-amyloidogenic pathway involves α- secretase and γ-secretase. β-secretase cleaves APP, generating soluble APPβ (sAPPβ) and a membrane-bound fragment C99. γ-secretase then processes C99, producing amyloid intracellular domain (AICD) and amyloid-beta (Aβ) peptides, which can aggregate into toxic oligomers and fibrils. Non-Amyloidogenic Pathway: α-secretase cleaves APP within the Aβ region, preventing Aβ formation. This generates soluble APPα (sAPPα) and C83. γ-secretase then cleaves C83, producing AICD and a short, non-toxic peptide called p3. (Hur, 2022).

2.3 Aβ and Tau Synergy

Aβ and phosphorylated tau tangles have been considered the hallmarks of AD for a long time. Their relationship has been considered temporal rather than synergetic, but recent findings highlight their interdependence in disease pathology. Several in *vitro* experiments have shown consistent results supporting the interdependence of Aβ and tau tangles. Human neural stem- cell-derived 3D culture systems with overexpression of mutant APP and PSEN1 (with no tau mutation) induced both Aβ and tau aggregation, tau following accumulation of Aβ (Lee, et

al., 2016). However, blocking Aβ in cultures through inhibition of either β- or γ-secretases prevented tau pathology, again demonstrating the necessary role of Aβ in the accumulation of tau tangles (Israel, et al., 2012). In the experiment where human brain-derived pathological tau (AD-tau) was injected in a wildtype mouse brain (no prior plaques), no AT8-positive DN was detected, suggesting the synergy between plaques and tangles for the formation of DN (He et al., 2018). Furthermore, study by Virginia Lee's group revealed that injection of AD-tau into 5xFAD mice leads to formation of AT8-positive dystrophic neurites (DN) around Aβ aggregates.

Another study showed that individuals experiencing progressive memory loss present with hypometabolism in the posterior cingulate cortex which is an area strongly linked to tau and plaque interaction (Zhang et al., 2021). Additionally, findings from the Alzheimer's Disease Neuroimaging Initiative (ADNI)—a longitudinal study focused on identifying AD biomarkers— indicate that tau-related cortical thinning is exacerbated in the presence of Aβ (Fortea et al., 2014).

In *vivo* experiments, in which synthetic or brain-derived Aβ was injected into the cortex or hippocampus of P301L-mutant tau mice, showed accelerated tangle formation both at the injection site and synaptically connected areas (Bolmont et al., 2007). The same trend was observed when human brain-derived paired helical filaments (PHF major tangle component) were injected into the brains of APP/PSEN1 mice; enhanced tau propagation was observed compared to wild-type mice (Vergara et al., 2019). 3-Tg mouse model expresses both tau and plaques, but plaques form and aggregate before tangles. Antibodies directed against Aβ plaques reduced early-disease but not late-disease tau formation (Oddo, S. et al., 2003).

Overall, independent sets of *in vitro* and *in vivo* experiments have consistently confirmed that Aβ plaques are key to creating the environment that promotes tau aggregation leading to the disease progression.

Conversely, tau has been shown to contribute to enhanced toxicity of Aβ, leading to more rapid deterioration of cognitive and motor phenotypes. For instance, tau knockout mice showed rescued cognitive performance and reduction in plaque load by 50% (Leroy et al., 2012). On the other hand, reduction of the endogenous tau levels did not alter plaque load, yet it still reversed memory impairment and decreased mortality (Nisbet et al., 2015; Roberson et al., 2007). Mechanistically Aβ and Tau can both interact with key proteins such as Fyn and NMDA receptor leading to Ca^{2+} influx and subsequent neuronal death. Aβ toxic oligomers bind to cellular prion protein (PrP) which further activates Fyn kinase and phosphorylates tau via NMDAR GluN2B subunit causing Tau aggregation. Additionally, Tau showed to independently influence NMDAR through binding to Fyn and allowing its interaction with postsynaptic density protein (PSD) forming a complex that exacerbates Aβ initiated cytotoxicity. Further supporting the influence of tau on Aβ, absence of tau decreases Aβ induced toxicity through Tua-Fyn-PSD mentioned mechanism (Zhang et al., 2023).

Taken together, Aβ appears to initiate a pathological loop in which tau amplifies neurodegeneration. This cycle ultimately accelerates disease progression, as tau becomes a key mediator of Aβ-induced toxicity. Therefore, while Aβ plaques facilitate tau aggregation; tau aggregation could also promote Aβ formation, further accelerating disease progression.

2.4 Microglia Involvement in Alzheimer's Disease Pathology

Microglia are myeloid cells that coordinate immune responses in the brain, playing a crucial role in maintaining brain health. Two broad categories of microglia are classical (M1) and alternative (M2) types where the former provokes inflammation and neurotoxicity, while the latter drives anti-inflammatory and reparative responses (Tang and Le, 2016; Colonna and Butovsky, 2017). The recently discovered subpopulation of microglia is disease-associated microglia (DAM) that surrounds the plaques in AD and other neurodegenerative brain diseases. Although the role of microglia in AD has not been fully established, some findings suggest that it might play a role in compacting plaques when surrounding neuritic Aβ plaques. An important aspect of the microglia-plaque interaction is the mechanism through which microglia detect Aβ plaques. Two TAM receptor tyrosine kinases, Axl and Mer, have been identified as key players in the identification of Aβ plaques, leading to their subsequent organization and phagocytosis.

Specifically, Axl mRNA has been shown to be overexpressed in disease-associated microglia (DAM) during the later stages of AD. Studies using double knockout ($Axl^{-/-}$ and $Mer^{-/-}$) mice demonstrated a reduced level of dense-core plaques and impaired Aβ plaque detection and organization (Huang et al., 2021). This supports the notion that microglia are primarily focused on protecting the brain from the toxic effects of Aβ plaque deposition, by actively engaging in the clearance and compaction of Aβ plaques and thus limiting the harmful impact of plaques on neurons.

Triggering receptor expressed on myeloid cells 2 (TREM2) is one of the risk factors for developing sporadic AD also known as LOAD, emphasizing the importance of microglial response in AD brains. TREM2 controls key microglial roles, such as phagocytosis, migration, lysosomal degradation and metabolism. In the context of AD, it is required for Aβ plaque compaction and neuronal health, highlighting microglia-neuron interaction and microglia-driven response to plaque accumulation (Van Lengerich et al., 2023). Additionally, this receptor is involved in switching microglia cells from the basal homeostatic to a disease-associated state.

TREM2 deficiency results in lower expression of several genes responsible for microglial activation, including inflammatory cytokines, trophic factors, and proteins related to phagocytosis (Czapski & Strosznajder, 2021).

Specifically, DAM in AD has shown to undertake two-stage transformation. Initially, homeostatic microglia shifts to Stage 1 DAM (intermediate stage) due to neurodegeneration- associated molecular patterns (NAMPs). Then, Stage 1 to Stage 2 DAM transition is modulated by TREM2 upregulation (Fig. 4). The gene suspected in maintaining the microglia in Stage 2 is Apolipoprotein A (APOE) that is involved in the autocrine or paracrine loop and upregulated in AD brains (Samant, Standaert, & Harms, 2024). This TREM2-APOE pathway seems to play a critical role in the transition and maintenance of DAM. Reduction in TREM2 results in decrease of apoptotic neuron clearance (Takahashi et al., 2005). Complementary reduction or deletion of APOE leads to reduced DAM signature in the disease brain (Song and Colonna, 2018). Hence, DAM might be potentially involved in plaque-debris clearance, however their role does not seem to be uniform, but instead AD stage dependent.

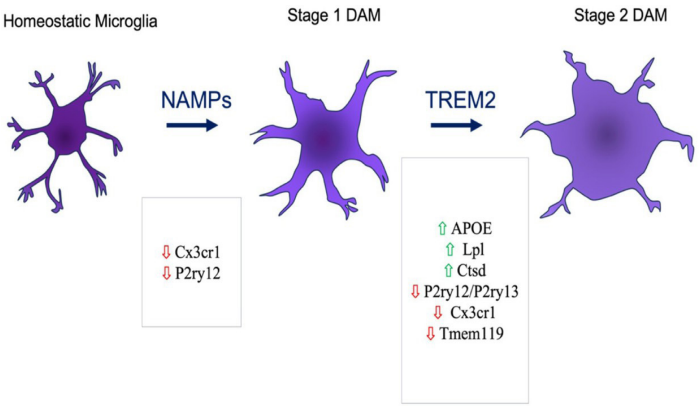


Fig. 4. **Microglial Transition into Disease Associated Microglia (DAM).** NAMPs regulate the first stage transformation through downregulation of homeostatic genes. The second stage transformation is caused by TREM2 signaling being a key component for final change. This process is accompanied by upregulation of some and downregulation of other homeostasis related genes. (Samant, Standaert, & Harms, 2024).

3. Behavioral Symptoms

Annually, 10-15% of adults (> 65 years old) diagnosed with Mild Cognitive Impairment (MCI) develop AD (Alzheimer's Association. n.d.). MCI is defined as an in-between stage between healthy aging and dementia characterized by memory, language, and other cognitive impairments with no adverse effects on daily life activities (National Institute on Aging, n.d.). Hence, it is crucial to study the contributing factors and characteristics of each stage of AD progression including MCI for timely pharmacological and behavioral interventions. It is worth noting that MCI has shown to be reversible when adopting a healthy lifestyle such as performing cognitively challenging tasks (Gates et al., 2010), eating healthy food (Lee et al., 2013), and exercising (Geda et al., 2010). The reversibility once again highlights the importance of understanding the timeline of the disease progression and its triggering factors.

3.1 Cognitive Behaviors

Since memory loss is a hallmark of AD-related cognitive deterioration, investigating its role in both pathological and normal aging is essential. By comparing memory function in healthy aging and AD, researchers can distinguish cognitive decline caused by the disease from age- related changes, helping to uncover underlying mechanisms and identify potential intervention targets.

Working Memory (WM) is key to maintaining and manipulating short term memories, contributing to decision-making and general cognition. The brain areas involved in formation of working memory—identified using positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) scans—include prefrontal cortex (PFC), parietal regions, cingulate gyrus and hippocampus (Kirova, Bays, & Lagalwar, 2015), all highly affected areas in AD patients. It is worth noting that it is natural to observe deterioration of WM and other cognitive functions associated with weakened connectivity among the mentioned areas in aging brain. A study comparing brain activity in young and older individuals performing the same cognitive task found an age-related increase in the recruitment of brain areas that are less task specific to account for weakened brain connectivity (Martins, Joanette, & Monchi, 2015; Poirier et al., 2021).

However, this altered brain activity pattern becomes even more pronounced when comparing individuals with mild cognitive impairment (MCI) and early-stage AD. A recent study shows a strong positive relationship between functional connectivity of the brain and AD progression marking the important effect of disease on brain connectivity (Carrasco-Gómez et al., 2024). Additionally, evidence provides that along with brain changes there are several aspects of cognition showing strong deterioration. Specifically, divided attention tasks reveal key differences between these groups showing episodic memory deficits in addition to WM in the early stages of AD. As the disease progresses, individuals exhibit worsening manipulation skills (memory recollection), failures in inhibiting irrelevant stimuli, and declines in selective attention. Since cognitive impairment is one of the first preclinical signs of AD, individuals with the poorest cognitive performance at the MCI stage are more likely to develop AD, while others have a greater chance of recovery (Kirova et al., 2015).

While decision-making in AD is relatively understudied, it remains a critical aspect of the disease alongside memory decline. It is central to the question of whether AD patients can actively participate in their treatment planning and make independent decisions. Santos et al. (2022) define decision-making as a complex mental process consisting of four components: 1) the ability to store, recall, and understand the meaning of information; 2) the ability to apply information in a relevant context; 3) logical thinking and mental comparison; and 4) the expression of choice and the maintenance of that choice until completion. This paper evaluates decision-making ability in relation to cognition and clinical factors (such as quality of life and awareness about the disease) in AD patients. The results suggest that while cognition is the major contributing factor, it is not the sole determinant, emphasizing the importance of using various measures to assess decision-making ability in AD patients (Santos et al., 2022).

Furthermore, curiosity is a basic cognitive factor encoded in our daily behaviors and conserved across species. There are numerous benefits to displaying curiosity, which drives learning and motivation—both of which are key to survival. A diminished sense of curiosity or information- seeking behavior is often linked to depression and apathy, common traits in AD patients (Kidd & Hayden, 2015). Due to the vagueness of the term, there is no specific definition of curiosity, and the subject of focus varies across labs and studies. For instance, curiosity can range from a "desire to respond to trivia questions" to the "strategic deployment of gaze in free viewing" (Gottlieb et al., 2013).

Most studies on human curiosity operationally define it as a preference for directing gaze toward novel, unfamiliar, or irregular objects. The available data provides evidence for diminished exploratory behaviors in AD patients in the later stages of the disease, as measured by exploratory eye movement (Daffner et al., 1992). In contrast, healthy subjects tend to devote more attention and time to watching novel or unusual objects, displaying signs of curiosity and exploratory behavior (Daffner et al., 1994). More recent studies have shown an age dependent decrease in exploratory behavior using various more sensitive measures and in various tasks (Mata et al., 2013). Hence, exploratory behavior appears to be dynamic throughout human life, suggesting AD may have an additive effect on exploratory behavior deterioration in aged individuals.

Similar to humans, a pronounced effect of age on exploratory behavior was observed in mice. Studies on aging mice reveal a tendency to repeat choices and limit exploration in various decision-making tasks (Hwang et al., 2023). For instance, when given multiple options, older mice exhibit a strong preference for sticking with prior choices, even in the absence of a reward. This highlights a shift in decision-making strategies with age. There is no conclusive data in AD mice, except for one recent study that investigated exploratory behaviors in mice by measuring active whisking behavior—a sign of healthy curiosity in rodents. Surprisingly, the results showed no genotype effect on exploration when comparing 5xFAD to control mice at 6- 7 months of age (Grant et al., 2020).

3.2 Non-Cognitive Behaviors

Motor impairment is a non-cognitive aging phenotype reliably associated with AD development (Buchman et al., 2020). Recent studies showed that motor dysfunction precedes MCI by several years (Yu et al., 2019) potentially being a clinical marker for MCI and AD. Beeri et al. (2021)conducted a nested substudy of 1,160 aging individuals from three longitudinal studies, assessing baseline motor activity and tracking changes over a seven-year period with annual cognitive check-ups. The results showed that better motor performance at a baseline (hand dexterity, hand strength, gait function) correlated with a reduced risk of developing MCI, and hand strength was also independently related to AD.

Gait impairment provides insight into interplay of the cognitive and motor components in AD patients enabling a better understanding of complex behaviors. A study by Kim et al. (2025) on gait impairment offers a novel, more in depth analysis of this specific motor function in relation to cognition and its role in tracking AD development. This study suggests that gait is an example of goal-oriented behavior that depends on various cognitive functions for its effective execution. In support of the positive relationship between gait and AD progression, Aβ plaque deposition has been shown to contribute to the deterioration of gait in AD patients (Del Campo et al., 2015). Additionally, this study identified the correlation between gait velocity and cortical atrophy in two major brain networks, each associated with distinct cognitive functions: default mode (DMN) and salience (SN) network in AD patients. According to a comprehensive review of DMN research by Menon, V. (2023), DMN is a collection of various regions that are active during the resting state to consolidate and process information. It is also involved in internally focused thought processes (self-reflection, daydreaming, mind wandering, recall of personal experiences) which constitute semantic and episodic types of memories. SN on the other hand is active when choosing which external stimulus needs to be attended, acting

as a switch between DMN and other brain networks required for performing a specific task (Schimmelpfennig, 2023) and plays major role in working memory (Fox et al., 2005). Given these functional roles of DMN and SN, atrophies in those two networks in AD may underlie a large range of altered behavior spanning cognition, decision-making and motor skills in AD patients.

4. Current Therapies and Therapeutic Approaches

There are limited treatment options for AD patients, urging the development of more efficient therapeutics that can at least slow down the disease progression in patients. The currently available drugs focus on the Aβ plaque clearance after cleavage or regulating acetylcholine and glutamate levels in the brain at different stages of the disease. Lecanemab (Lequembi) and Donanemab (Kisunla) are the two currently available treatments for Aβ clearance in the brain of MCI presenting AD patients (needs to be diagnosed in early stages). Some of the side effects are changes in vision, confusion, dizziness, headache, nausea, or seizures. People with moderate symptoms of AD are usually prescribed with Galantamine (Razadyne), Rivastigmine (Exelon), and Donepezil (Aricept) which inhibit enzymes breaking down acetylcholine, increasing the level of this neurotransmitter improving cognition and memory. These drugs are usually well tolerated, and the side effects range from nausea, vomiting, loss of appetite, and increased frequency of bowel movements. Lastly, Memantine (Namenda) is a drug prescribed for individuals with moderate to severe AD. It blocks NMDA receptors in order to prevent glutamate-induced cytotoxicity in the hyperexcited cells observed in AD patients. Here are some of the well-known side effects: headache, constipation, confusion, and dizziness (Alzheimer's Association, n.d.).

Alternative non-drug approaches for slowing down disease progression have been recently developed. Sovrea et al. (2025) provides a comprehensive review of the currently used non- drug treatment including focused ultrasound (FUS) and transcranial pulse stimulation (TPS). Both of which are non-invasive: TPS sends ultrashort ultrasound pulses, also known as shockwaves, to targeted small brain regions (Cont et al., 2022) whereas FUS uses acoustic waves. The primary limitation of FUS is the brain depth it can reach, and it needs to be paired with MRI for precise targeting (Krishna,Sammartino,Rezai, 2018). Microglial activation, synaptic plasticity and other physiological changes are amongst some of the FUS benefits (Fig. 5A). TPS has shown significant improvement in cognition through morphological and functional changes in the brain (Fig. 5B) (Sovrea et al., 2025). A study by Nazarian, Yashin, & Kulminski (2019) showed that repeated TPS sessions lead to sustained cognitive benefits in memory retention and executive function. Although these methods are promising, there is still a need for further research into their long-term effectiveness

5. Animal Model Research

Alzheimer's disease is a complex condition with unclear, multifactorial causes limiting our ability to effectively prevent its development. One of the advantages of in vivo research over in vitro in the context of AD is the ability to study the complex interplay of various factors involved in the development of pathology. *In vitro* studies, using isolated cells, offer a more simplified view of the disease process, often disregarding the intricate interactions between different cell types, and brain regions that occur in the human brain. In contrast, *in vivo* models allow for a more holistic approach, capturing the dynamic interactions and systemic changes that are crucial for understanding AD pathology (Drummond & Wisniewski, 2017).

After the discovery and establishment of the "amyloid cascade hypothesis" stating that Aβ is the central component of plaque in AD in 1984 (Glenner & Wong, 1984), the challenge was to develop an effective model to study the hypothesis further. The first major milestone in developing the AD model was made in 1995 when PDAPP was developed. This model expressed one APP mutation accompanied by memory loss at the age of 3 months, and plaque accumulation by the age of 7-8 months (Yokoyama et al., 2022). This was a breakthrough for *in vivo* AD research, leading to the development of the current 50 mouse models for AD (*MODEL- AD*, 2023).

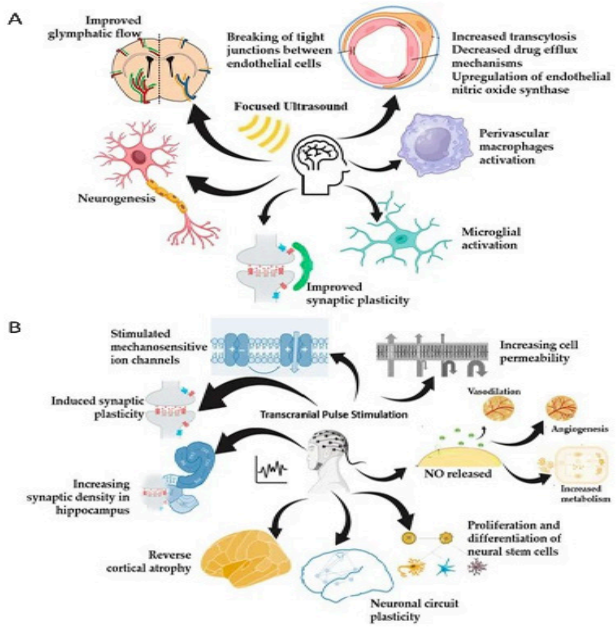


Fig. 5. **Benefits of Focused Ultrasound (FUS) and Transcranial Pulse Stimulation (TPS).**
(**A**) The benefits of FUS as a non-drug, non-invasive therapy for AD Some of the key benefits include microglial activation, leading to an increased rate of amyloid plaque clearance, neurogenesis to compensate for extensive neuronal loss, enhanced blood-brain barrier (BBB) permeability, and modulation of neuronal activity to restore disrupted circuits.(**B**) The benefits of TPS as a non-drug, non-invasive, and novel therapy for AD. Some of the key benefits include increased synaptic density in the hippocampus, leading to improved hippocampal function, reversal of cortical atrophy, aiding in the restoration of damaged brain regions, and enhanced neuroplasticity, potentially slowing disease progression. (Sovrea et al., 2025).

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Currently, most AD research focuses on identifying clinical biomarkers for effectively detecting and predicting the disease progressions on one end and developing efficient model systems for uncovering its underlying mechanisms on the other end. Animal models are essential for the latter effort, as they provide a complete system where intricate disease processes involving organically connected cell types, brain regions, and organs can be investigated. Many AD animal models have been developed to replicate some pathological physiology and behavioral symptoms observed in human AD patients, but no single model recapitulates the comprehensive and heterogeneous nature of human AD. Thus, it is important to determine which aspects of human AD can be properly investigated in each model.

On the other hand, 6xTg can be used to test therapies involving tau related gene inhibition to observe its effect on Aβ plaque production and vice versa. 5xFAD is frequently used to study the distribution and accumulation of amyloid plaques. This model provides insights into the mechanisms underlying plaque pathology, one of the hallmark signs of AD, often in the absence of tau pathology. Alternatively, models like 3xTg can be used to study secondary AD symptoms involving loss of olfaction, which is one of the initial preclinical symptoms in AD patients.

Behavior is another aspect crucial to evaluate in mouse models as AD patients typically exhibit non-cognitive symptoms before cognitive impairments emerge, making it crucial for understanding early-stage AD. While all the models mentioned are used for behavioral evaluations, the current lack of consistent results highlights the urgent need for more effective and replicable methods of behavior and cognition assessment in AD research.

5.1 Mouse Model: 5xFAD

5xFAD mice express five familial mutations in APP (Swedish

K670N, M671L), (Florida I716V), (London V717I), and PSEN1(M146L, L286V) genes, but no neurofibrillary tangles are observed in this model (Oblak et al., 2021). Plaque accumulation starts at the age of 2 months and continues with age mimicking human pathology. The transgenes were implemented under the control of the Thy1 promoter (sensitive to progesterone) to ensure the expression specifically in neurons to mimic the localization observed in humans (Jankowsky et al., 2017). Based on these findings, we expect female mice to have a more deteriorated cognition and behavior as well as more plaque accumulation compared to the age-matched male mice. Visual (Wang et al., 2017) and olfactory (O'Leary et al., 2020; Lenoir et al., 2019) functions have been examined across different studies but have not shown any consistent results. Substantial research has also been performed in spatial memory of 5xFAD mice using Morris Water Maze (MWM) Test that showed significant learning impairment and latent escape as early as 5 months of age (Tang et al., 2016). O'Leary, et al. (2020), on the other hand, did not see memory impairment till 12-15 months confounded by motor impairment. One of the major gaps in research using 5xFAD mice is the lack of robust cognitive characterization which is key in understanding biological processes underlying behavioral deficits and thus critical for evaluating the effectiveness of potential therapeutics (Padua et al., 2024). Inconsistent results across studies can be potentially introduced through differences in housing, food consumed, or types of tests used to measure those factors. Besides inconsistent results in cognitive and behavioral deficits, the limitations of 5xFAD model include the lack of other pronounced physiological parameters such as tau tangle formation. Nevertheless, this model is attractive due to the strongly expressed plaques in the brain as well as the simplicity of breeding.

5.2 Mouse Model: APP/PS1 KI

APP/PS1 KI model was developed by inducing several point mutations in APP and PS1 genes, namely: M233T and L235P in PS1 and the London (V717I) and Swedish (K670N/M671L) in APP under the control of the Thy1 promoter (*APP751SL/PS1 KI | ALZFORUM*, n.d.). APP/PS1 Ki is a rare type of transgenic mouse model for studying AD that expresses neuronal loss along with amyloidosis which are not as common in other models. The neuronal loss starts at the age of 6 months and worsens gradually with age (Faure et al., 2011). Despite the pronounced pathological changes in the brain, there are conflicting ideas regarding the non-cognitive and cognitive changes in this mouse model. Initially, cognition was found to deteriorate at the ages 7-8 months with no pronounced non-cognitive changes (Serneels et al., 2009, Radde et al., 2006). Later findings stated that APP/PS1 KI demonstrates memory impairment as early as 6 months (Faure et al., 2011), while the most recent data shows those changes in age of 11 months (Webster et al., 2013). A paper evaluating the effect of aerobic exercises on cognition of 4-month-old APP/PS1 KI mice has showed decline in spatial memory at the baseline and significant improvement because of consistent exercising (Wang et al., 2024). Anxiety on the other hand did not show significant differences compared to the wildtype littermates (Webster et al., 2013). All in all, this mouse model shows latent cognitive and almost no non-cognitive changes compared to other AD models. However, it is very effective in studying neuronal loss and plaque accumulation following AD pathology development. Due to the limited intrinsic behavioral changes, this model might provide insight into the effect of external factors such as stress induced behavior shift in the presence of AD markers.

5.3 Mouse Model: 6xTg

To address the challenges of accurately representing AD in mouse models and exploring the interplay between tau and amyloid plaques, researchers have developed models expressing both plaques and tangles. These dual-model systems will potentially provide a more comprehensive understanding of the pathological, cognitive, and behavioral changes associated with AD, offering insights closer to the human condition.

This is a relatively new model for studying (developed in 2021) AD that aims at expressing both plaques and tau tangles by crossbreeding 5xFAD and JNLP3 (overexpresses MAPT mutation inducing aggressive tangle production) (Uras et al., 2023). This model effectively expresses various pathological AD features including plaque formation, abnormal tau phosphorylation, neuronal loss and astrocyte activation (Tag et al., 2022). Behaviorally, this model shows heightened anxiety and depression like state as well as hyperlocomotion at ages 9-11 months. Memory impairment is observed at around the same age (Tag et al., 2022), which contradicts earlier findings claiming memory decline to happen at 2 months of age (Kang et al., 2021).

Although deemed effective, this model has very limited information available on the non- cognitive and cognitive changes. Only a few studies provide insights into the onset of non- cognitive impairments, and thus a comprehensive understanding of their progression remains lacking. Moreover, conflicting reports on the timing of cognitive decline emphasize the need for further studies to establish a clearer timeline of behavioral and functional impairments in this model.

5.4 Model: 3xTg

The 3xTg mouse is characterized by mutation of three genes APP, PSEN1, and Tau, and therefore expresses both amyloid beta and tau tangles. This model is useful for studying the correlation between amyloid beta and tau tangles and it also effectively shows cognitive decline that starts at the age of 6 months and progressively worsens with age (Belfiore et al.,2019). 3xTg mice express mild cognitive deterioration with the Barnes Maze being the most sensitive measure of cognition (Kurt et al., 2015). One of the preclinical symptoms of AD is a loss of olfaction, which can be measured in this task. According to the buried food test findings, female mice spent a significantly longer time looking for food compared to the male, the latency to find the food also deteriorated as the mice aged, showing age-dependent loss of olfaction (Mitrano et al., 2021). The strength of this model is the expression of both hallmark signs of AD that allows us to study the relationship between Aβ and tau tangles. Additionally, mild cognitive decline is observed at 6 months of age making it an effective model to study cognition and pathology interaction in AD. The major disadvantages of the model, however, are the lack of neuronal loss regardless of plaque and tangles expression and variability among the colonies (Zhong et al., 2024). The last was likely caused by genetic drift that took place in this model population introducing phenotypic heterogeneities.

6. Assessment of Behavioral Symptoms in AD Mice

Studying AD phenotypes in mice requires a variety of behavioral and histological assessments. The collection of behavioral data is crucial because histological analysis is only obtained after sacrificing mice. Additionally, human AD patients start demonstrating behavioral and motor deficits before cognitive symptoms become apparent. Non-cognitive symptoms referred to as Behavioral and Psychological Symptoms of Dementia (BPSD) highlight affective dysregulation, with apathy and depression being the most prevalent symptoms (Selles et al., 2018). Similarly, a study conducted using Alzheimer's Disease Assessment Scale (ADAS-Noncog) identified tremor, depression, psychotic symptoms as the most prominent non-cognitive symptoms in AD patients (Fernández et al., 2010). Additionally, anxiety is commonly seen in individuals with MCI before the start of AD as well as considered a contributing factor to a more rapid transition from MCI to AD (Mendez, 2021). Detection and treatment of early symptoms might be an effective strategy to slow down disease progression. Hence, a thorough characterization of behavioral phenotypes—such as speed of mouse movement, gait, and freezing behavior—is essential for evaluating the effectiveness of mouse models in replicating non-cognitive aspects of AD in humans, uncovering the underlying pathophysiology of behavioral deficits in AD, and assessing the efficacy of therapeutic interventions.

6.1 Open Field Test

The open-field test (OPT) is a widely used assay that examines the animal's free locomotor activity and exploratory behavior. During an open-field test, mice are placed in the center of the open-field arena and left to roam freely for 5-10 minutes while the video is being recorded with an overhead camera. The usual kinematic variables extracted from the video include velocity, total distance, and time spent

in the center compared to the time spent in the periphery of the arena.

In addition to video recording, an infrared (IR) beam brake system may be utilized for monitoring voluntary locomotor activity 24/7 mostly used for home cages under different light brightness (Klein et al., 2022). It is an effective tool to measure behaviors such as rearing and climbing; it can also be used to measure trajectory, distance traveled, and position distribution. However, it is not effective in studying social interactions involving multiple mice in the same cage. Although both tracking methods are effective, video tracking allows analysis of more complex behaviors and can be used for a wider variety of analyses compared to the IR beam. The open field test is also used for measuring anxiety in mice. Healthy mice tend to acclimate to the environment and as the level of anxiety decreases, they spend more time in the center compared to the periphery (Carter & Shieh, 2015). However, it is not the most effective measure because due to the rodents' innate fear of predation, mice naturally spend less time in the open space of the arena corresponding to its center (Pentkowski et al., 2021).

A paper characterizing 5xFAD mouse model report that the distance traveled as well as velocity shows significant decline at the age of 18 months. Younger mice of 8 months exhibit a greater preference for the center of the arena compared to the control group, indicating reduced anxiety-like behavior (Forner et al., 2021). A significant reduction in locomotor activity has also been observed between 4-month-old and 6-month-old ages in 5xFAD mice, compared to control in both males and females (Poon et al., 2023). Taken together, OFT revealed alterations in some non-cognitive functions in 5xFAD mice.

6.2 Morris Water Maze Test

The Morris water maze (MWM) test is one of the most commonly used methods for evaluating short-term and long-term spatial memory in mice. Most papers in the field use the MWM results as a way to measure memory, learning, and motor activity while swimming. 5xFAD mice regardless of their sex show deterioration in learning at age 6-9 months and only worsened in older mice, with co-occurrence of locomotor dysfunction starting at the age of 9 months.

Memory deterioration however did not occur until age 12-15 months where females showed inconsistent performance between ages 9-12 months providing inconsistent evidence (O'Leary & Brown, 2022). Another paper analyzing the data from TG-2576 (AD model) mice discusses the appearance of cognitive deficit between 12-18 months requiring a large sample size, suggestive of a small effect size (Choi et al., 2023). As such, despite its wide popularity, MWM test shows inconsistencies in determining the age of onset for cognitive and behavioral deficits. Other disadvantages of this test include induced stress in mice reflected in high cortisol levels, general unwillingness to be in water, and spatial learning variability (Othman, Hassan, & Has, 2022).

These may interfere with memory consolidation and overall performance of the mice, a confounding effect between cognition and emotion. Recently published papers have offered to optimize MWM for a more reliable evaluation of discussed behaviors (Bailoo et al., 2024). To sum up, MWM is an important method for evaluating an array of cognitive and behavioral deficits but requires some revisions to improve the consistency and robustness of results.

6.3 Elevated Plus Maze Test

The Elevated plus maze (EPM) test is used to measure the level of anxiety in AD mice depending on the time they spend in an open arm versus a closed arm. Naturally, closed arms are associated with safety, and open arms tend to induce anxiety. Therefore, the more time a mouse spends in the open arms, the lower its anxiety is considered to be. However, EPM studies show inconsistent results on 5xFAD mice. Some papers claim 5xFAD mice to have decreased anxiety (Forner et al., 2021), whereas the majority of the papers indicate no changes (Flanigan et al., 2014). Making it further confusing, a minority of studies found increased anxiety (Dong et al., 2020, Locci et al., 2021). The widely different EPM results might reflect confounding effects of AD mice's increased sensitivity in their vibrissa (Grant et al. 2020). Because of the increased sensitivity in their vibrissa, AD mice engage in barbering behavior less often than control and would not permit control mice to barber for them. Furthermore, AD mice, when their whiskers were trimmed, showed an increased frequency of entries to closed arms of the EPM (Flanigan et al., 2014). Conversely, 5xFAD mice, with their intact whiskers, visit the closed arms less frequently because they avoid stimulating their over-sensitive vibrissa from touching the walls of the closed arms rather than they are less anxious. Therefore, EPM is an unreliable measure of anxiety in 5xFAD mice.

7. Major Knowledge Gap in AD Research

Despite decades of extensive research, significant gaps remain in our understanding and treatment of AD. One of the most urgent challenges is identifying the early mechanisms that trigger disease onset and their associated behavioral changes. Current therapies largely target downstream pathological features—such as amyloid plaques and tau tangles—often after irreversible brain damage has occurred. To enable earlier intervention and meaningful prevention, it is critical to uncover the upstream molecular and cellular events that initiate the disease process and their correlated behavioral diagnostic markers.

While substantial progress has been made in characterizing molecular pathology, relatively little is known about how these changes disrupt neural circuits and large-scale brain networks. A deeper understanding of how AD alters information processing at the systems level—using techniques such as in vivo electrophysiology, calcium imaging, and functional connectivity analysis—is essential for linking cellular pathology to behavioral and cognitive decline. Additionally, increasing evidence highlights the critical role of non-neuronal cells—such as microglia, astrocytes, and peripheral immune cells—in modulating disease progression.

However, the precise contributions of these cell types remain poorly understood. Detailed mapping of glia-neuron and immune-brain interactions throughout disease progression may uncover novel targets, particularly within the context of neuroinflammation.

Another unresolved question involves the mechanisms through which it contributes to functional neural disruption and behavioral symptoms remain poorly understood. While soluble Aβ oligomers are believed to impair synaptic function and alter network activity, it is still unclear how these molecular changes translate into specific cognitive and non-cognitive deficits. Moreover, the relative contributions of soluble versus insoluble Aβ species to disease progression are still debated.

Additionally, the mechanism of amyloid pathology interaction with other key factors—such as tau accumulation, glial activation, and immune responses—to drive the full clinical presentation of AD is still elusive. These uncertainties highlight a critical need to better define how amyloidopathy affects neural circuits and behavior, particularly in the early stages of the disease, in order to improve the translational relevance of animal models and guide the development of more effective therapeutic strategies.

Animal models are indispensable for tackling these challenges and advancing the development of preventive and disease-modifying therapies. However, a persistent issue is the disconnect between preclinical success and clinical efficacy. Many treatments that show promise in animal models ultimately fail in human trials. This discrepancy may stem from the heterogeneity and multifactorial nature of AD—both in its pathology and clinical presentation. Animal models often capture only limited aspects of the disease and may not reflect the complex interplay of risk factors and compensatory mechanisms present in human populations. As a result, therapeutics that target isolated features may fall short in treating the broader, more variable AD spectrum.

Bridging the gap between preclinical predictions and clinical outcomes requires a clearer understanding of the strengths and limitations of each animal model. Yet, behavioral characterization across AD models remains incomplete and inconsistent, making it difficult to relate specific pathological mechanisms to functional outcomes.

To address these gaps, a multidisciplinary approach that integrates molecular biology, systems neuroscience, immunology, and behavioral analysis is essential. Toward this goal, my thesis focuses on a detailed characterization of both cognitive and non-cognitive behaviors in the 5xFAD mouse –known to be a reliable model for plaque accumulation—and how these behavioral changes correlate with the plaque burden.

8. Present Study

Goals

A key unmet need in AD research is the ability to detect the illness at its very onset. Currently available therapies aim to slow amyloid-β plaque buildup—the first pathological event that ultimately drives widespread neurodegeneration—so their success depends on intervening before irreversible damage occurs. Unfortunately, no reliable behavioral readouts exist for these earliest stages, either in people or in animal models. This dissertation addresses this critical gap by developing behavioral biomarkers sensitive to the initial emergence of amyloid pathology. Using the 5xFAD mouse—an established model that rapidly and faithfully recapitulates plaque formation—we will comprehensively profile cognitive, affective, and motor functions across early to late phases of deposition. Unveiling how plaques influence both cognitive and non-cognitive domains could provide a foundation for early disease diagnostics biomarkers, improving the effects of the currently available therapeutics, and developing new disease-modifying interventions. The specific aims of this study are:

- Establish a clear timeline for cognitive and behavioral changes associated with AD progression in 5xFAD mice
- Explore the relationship between amyloid plaque burden and cognitive decline
- Investigate how the spatial distribution of plaque deposition related to specific behavioral deficits, such as impairment in motor coordination and anxiety-like behavior
- Assess how plaque location may contribute to disruption in neural network function, particularly those involved in decision-making and memory

Hypothesis

If amyloid plaques are sufficient to drive alternations in neuronal circuits and promote neurodegeneration, then 5xFAD mice should exhibit early onset of cognitive impairment, particularly in tasks that demand integration of multiple brain functions, including sensory processing, motor coordination, strategic action planning, and learning. To test this hypothesis, I will compare cognitive and non-cognitive task performance between 5xFAD mice and age- matched wild-type controls and investigate how performance metrics align with plaque pathology.

Methods

1. Animals

All procedures are performed in accordance with protocols approved by the Rosalind Franklin University Institutional Animal Care and Use Committee (IACUC) and guidelines of the National Institutes of Health (NIH). Male and female genetically modified 5xFAD and control wild-type mice, all from a C57BL/6J background, at two age groups (G1:2 months old and G2:5-7 months old) were used for behavioral and histological assays (Table 1). All mice are kept at 12 hours light/dark cycle with food at libitum. Behavioral assays were performed during the dark cycle. Estrous cycle in female mice is not tracked.

2. Headbar Implant Surgery

To enable head-fixation during the cognitive behavioral task described in the next section, mice first underwent surgery to implant a headbar. This procedure was conducted under aseptic conditions with the mouse fully anesthetized.

Age	Genotype		Sex	Age Group
	AD	Control		
2	H0930, H0931,H1506,H1507	H1522,H1555,H1556,H1557	F	G1
	H0928, H0929,H1504,H1505	H1519,H1528,H1558,H1559	M	
5	H1030, H1031, H1033	H1117, H1118, H1119	F	G2
	H1028, H1029	H1160, H1161	M	
7	H1034, H1027	H1156, H1116, H1115	F	
	H1025, H1026	H1153, H1163	M	

Table 1. Genotype, sex, and age of mice used in the study.

Anesthesia was initiated by placing the mouse in an induction chamber with 2% isoflurane in 100% oxygen until the heart rate slowed to approximately 15–16 beats per 15 seconds and the toe-pinch reflex was consistently absent. Once fully anesthetized, the mouse was transferred to a stereotaxic frame equipped with a heating pad set to 37 °C (Fig. 6A), and anesthesia was maintained using isoflurane delivered via a nose mask. To ensure preemptive analgesia, meloxicam (10 mg/kg) was administered subcutaneously prior to the surgical procedure. Eye ointment was applied periodically to prevent corneal drying, and lidocaine was applied to the ear bars to minimize discomfort during positioning in the frame.

The scalp was disinfected by alternating applications of 70% ethanol and iodine three times, followed by a midline incision. Bupivacaine was applied to the exposed skull for local anesthesia. The skin and underlying tissues were carefully retracted using a dental probe under a microscope, minimizing trauma and bleeding, which was managed with saline-soaked Surgifoam as needed.

After fully exposing the skull, the periosteum was removed with a scalpel until the cranial suture lines were clearly visible, providing a clean surface for headbar attachment. A brief application of dentin was followed by a saline rinse and ethanol drying. Medical-grade adhesive was then applied to the skull, and the headbar—aligned with the lambda suture line (Fig. 6B)— was positioned in place. The adhesive was allowed to cure for 20–30 minutes while a post- operative recovery cage was prepared with hydrogel and recovery diet gel.

Dental acrylic was used to seal the exposed skull and surrounding skin to protect against infection. The mouse's ID was marked on acrylic and sealed with superglue. To prevent dehydration, 0.5 ml of saline was administered subcutaneously at the end of surgery. Postoperative care included close monitoring of the mouse's health and pain levels. Meloxicam was administered subcutaneously for 48 hours following surgery at 12–24-hour intervals.

3. IBL Task

The International Brain Laboratory (IBL) task is a standardized decision-making task for mice, conducted using standardizing hardware, software, surgical, and training protocols to ensure reproducible behavioral and neurophysiological results across studies (IBL set up: Fig. 7A,B) (The International Brain Laboratory et al., 2021). This complex behavioral task engages various components of cognition, including learning, memory, motor functions, and sensory processing, all of which are crucial for decision-making. In this task (Fig. 8), mice are presented with a visual stimulus either on the left or right side of the screen and must turn a steering wheel either clockwise or counterclockwise, depending on the stimulus location, to receive a reward. At the beginning of training, only high-contrast (100% and 50%) stimuli are presented to facilitate

learning. If performance reaches 80% or more for three consecutive days, lower-contrast stimuli (25%, 12.5%, 6.125%, 0%) are gradually introduced. Hence, the highly sensitive and cognitively demanding IBL task provides a novel approach to assess how AD alters cognitive functions underlying complex goal-directed and learning behaviors.

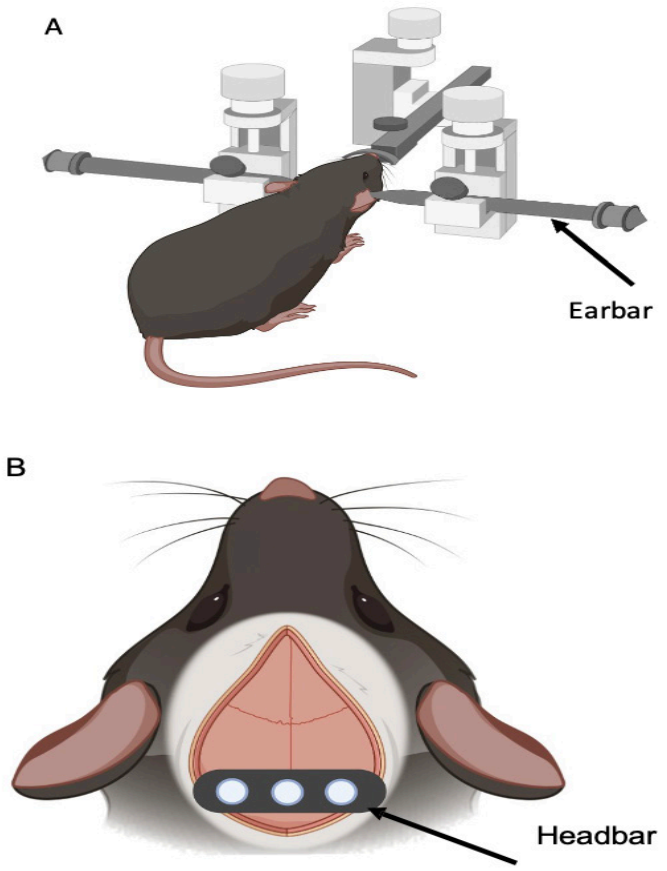


Fig. 6. The visual representation of the surgery. (A) Shows the set-up of a stereotaxic frame. One of the arrows points at ear bars that help fixate the head during the surgery and the other points at the headbar that gets implanted to the skull surface. (B) Shows lambda suture line on the skull where the headbar will be placed. Designed with BioRender.

3.1 IBL Training Timeline

Following a 7-day recovery period after headbar implantation, mice undergo water restriction, rig play, and habituation according to the IBL protocol prior to beginning the behavioral task.

Rig play consists of daily 5-minute sessions conducted over four consecutive days, during which no visual stimuli are presented. The primary goal of this phase is to acclimate mice to the experimental setup and foster an association between the behavioral rig and water reward delivery.

Next, habituation is carried out over three days with progressively increasing session durations: 15 minutes on Day 1, 30 minutes on Day 2, and 45 minutes on Day 3 (Fig. 9). During this phase, visual stimuli are introduced, and the wheel remains locked. The stimulus automatically moves to the center of the screen, triggering water delivery through the spout. This setup helps mice form an initial association between stimulus positioning and reward—a concept central to the upcoming task

During the task phase, mice are head-fixed and use the wheel to control the position of a visual stimulus presented on either the left or right side of the screen. A correct response—bringing the stimulus to the center within 60 seconds—results in water delivery. If the mouse fails to do so, the trial is aborted, and a white noise tone is played as negative feedback. Each trial is followed by a brief intertrial interval (~2 seconds), after which a new trial begins.

A session concludes under any of the following conditions: The mouse fails to complete more than 400 trials within 45 minutes; Training exceeds 45 minutes, and fewer than 45 trials are completed in the last 5 minutes; The total training time reaches 60 minutes. Each mouse performed one session of the IBL task per day for 30 consecutive days.

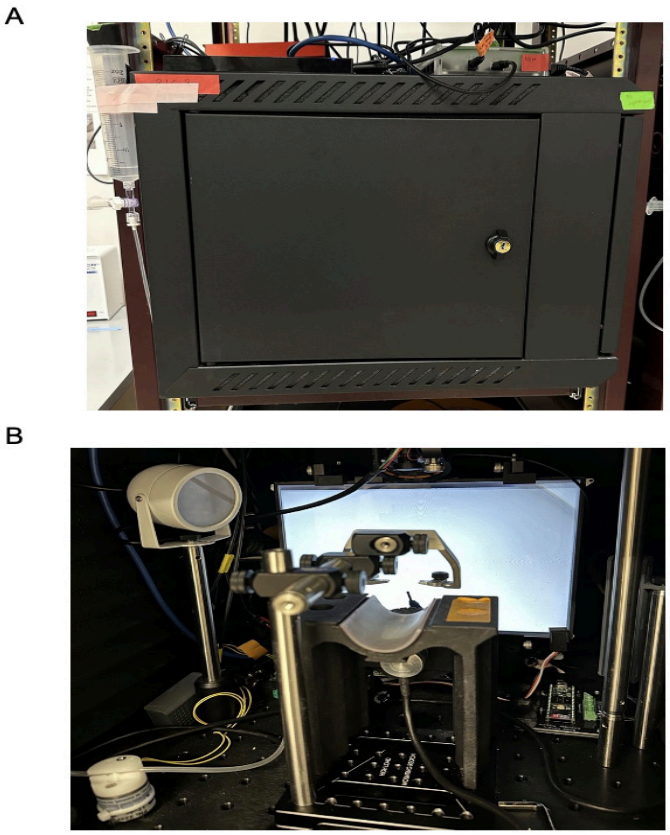


Figure 7. Standardized IBL Behavioral rig (A) external view (B) internal view

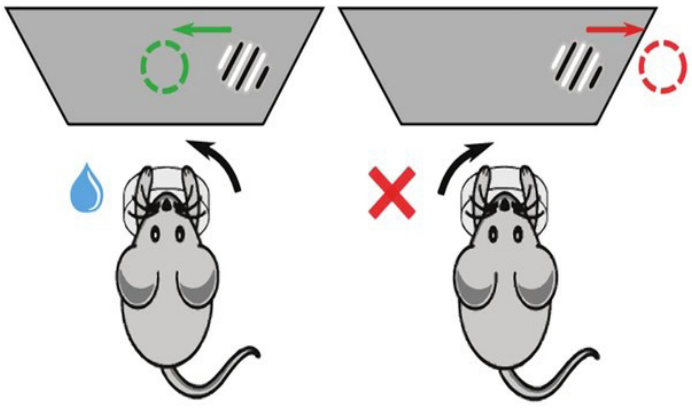


Figure 8. The visual representation of the IBL task. When the stimulus (black and white lines) is presented on the right side of the screen (gray rectangle) the wheel needs to be stirred counterclockwise (direction pointed by green arrow). Successful completion of the trial leads to reward (water) and wrong answer leads to aversive noise. (The International Brain Laboratory et al., 2021).

Figure 9. The timeline of Pre - IBL Task Steps. Each dot corresponds to one day. Created with BioRender.

4. Open Field Test

Open Field Test (OFT) is used to study free-roaming behavior in mice, from which non-cognitive brain functions such as emotional regulation (e.g., anxiety) and motor control can be assessed. The testing arena measures 50 × 40 × 33 cm (width × length × height). Before running the test, mice are acclimated to the testing room for at least 30 minutes in their home cage to minimize stress in the new environment. Each mouse was consistently placed in the lower right corner of the arena to standardize the starting position across subjects.

In this study, the duration of OFT is 30 minutes. Behavioral analyses focused on two-time windows: the first five minutes (0-5 minutes) and the middle five minutes (15-20 minutes), capturing early exploratory behavior and late-stage activity patterns. The entire session was recorded using an overhead camera (AV Alvium 1800 U_291, Allied Vision) at 60 frames per second, with video saved in mp4 format at a resolution of 1944 ´ 1472 pixels.

Although this test requires relatively minimal direct involvement from the experimenter as the recording and analysis pipelines are highly automatized, it does not entirely eliminate the potential risk for introducing experimenter's bias. The major source of bias can be introduced through pre-test handling and the way the mice are placed in the testing zone. To minimize these risks, in this study, only three experimenters handled the mice in all OFT sessions, each following a standardized protocol to ensure high procedural consistency across animals.

5. Methoxy-X04 Stain Preparation and Injection

Methoxy-X04 is a stain derivative of the widely used Congo red, known for labeling amyloid plaques. It crosses the blood brain barrier and selectively binds to fibrillar β-sheet deposits with a binding affinity of Ki = 26.8 nM (Methoxy-X04, n.d.). The methoxy-X04 solution is prepared following a published protocol (Bisht, El Hajj, Savage, Sánchez, & Tremblay, 2016). A stock of 10 mg methoxy-X04 powder is first dissolved in dimethyl sulfoxide (DMSO) solvent, then mixed with glycol and saline to create a final 2 mL solution at a concentration of 5 mg/mL. The solution is incubated overnight to reach the desired state. Mice are injected intraperitoneally (IP) at a dose of 10 µg/g body weight.

6. Perfusion and Brain Sectioning

Approximately 24 hours after the Methoxy-X04 injection, mice undergo transcardial perfusion for tissue fixation and brain extraction. Deep anesthesia is induced using a ketamine/xylazine mixture (100 mg/20 mg/kg, IP), and full anesthetic depth is confirmed by the absence of a response to a firm toe pinch.

Perfusion is carried out using 1× phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA). PBS (20–25 mL) is used first to flush out the blood, ensuring cleaner tissue and clearer visualization of the brain, while PFA (20–25 mL) serves as a fixative to preserve tissue architecture and facilitate downstream extraction.

After perfusion, the brain is carefully extracted and post-fixed in 4% PFA for at least 24 hours. It is then transferred to a 30% sucrose solution for cryoprotection and stored for a minimum of 48 hours. Prior to sectioning, the brain is positioned in a brain matrix to remove the cerebellum and flatten the base. This ensures stable placement of the brain on the sliding microtome stage (Leica SM2000). The microtome is pre-chilled, with dry ice arranged around the center of the stage for rapid freezing and maintenance of temperature at ~-20°C. For histological analysis, coronal brain sections are collected at a thickness of 50 µm.

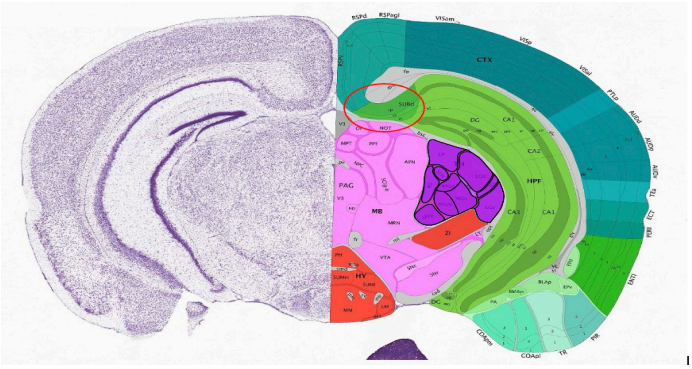


Figure 10. Selected tissue slice for histology analysis. The target coronal section for plaque quantification includes subiculum (SUB; marked with red circle) and hippocampus (HPF; the larger light green portion below SUB). (Allen Institute for Brain Science, n.d.).

8. Data Processing

All numerical data analyses and visualizations in this study (e.g., task performance in the IBL task, total distance travelled in the OFT, and statistical analyses) were performed using custom Python code. The analysis pipelines utilized open-source libraries, including SciPy, Matplotlib, NumPy, Pinguin, and Pandas.

8.1 IBL Task Data Analysis

Task performance in each session is quantified as the fraction of correct trials. To assess learning over the course of 30 daily sessions, the task performance is plotted as a function of training day and this plot is referred as learning curve. To examine learning dynamics in discrete phases, the training period is divided into three phases—initial (days 1–5), middle (days 16–20), and final (days 26–30)—with each phase comprising five days of data. The median performance across these five days is calculated for each mouse when comparing across different experimental groups (e.g., 5xFAD versus control, young versus older) in each phase.

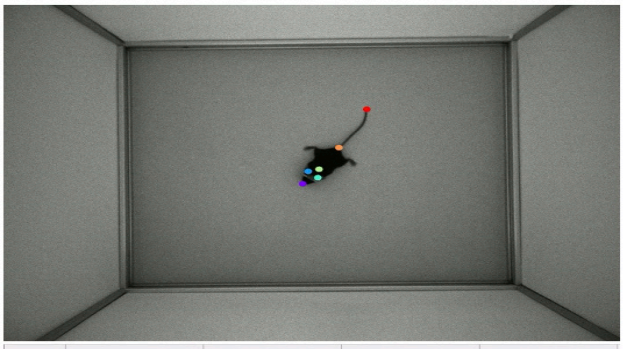
To quantify the decision-making strategies of individual mice, each trial is categorized based on the relationship between the current choice and the choice and outcome of the previous trial. If the choice in the current trial is the same as the choice in the previous trial, the trial is labeled as a "stay"; If it differs, it is labeled as a "switch". A "win/switch" is defined as a switch following a successful (rewarded) trial, while a "lose/switch" refers to a switch following a failed (unrewarded) trial.

8.2 OFT Data Analysis

The video data recorded during the OFT is first processed using DeepLabCut (DLC), an AI-based markerless body-part tracking software (Nath et al., 2019). DLC is pretrained to track six distinct anatomical landmarks on a mouse in each frame (Fig. 11A)—snout, left ear, right ear, nape, tail base, and tail tip—so that it can automatically detect these body parts from each video frame and outputs their x- and y-coordinates in pixel units.

The input to DLC is the mp4 video file from the OFT session, and the output is a spreadsheet containing the x and y coordinates of the six body parts for every frame of the video (Fig. 11B). DLC's "filtered predictions" feature is applied, which smooths the trajectory data and reduces noise. For locomotor analysis in this study, the "tail base" coordinates are used.

A



B

	scorer	DLC_resnet50_Park...	DLC_resnet50_Park...	DLC_resnet50_Park...
1	bodyparts	Tail_Base	Tail_Base	Tail_Base
2	coords	x	y	likelihood
3	0	1469.9541015625	1127.156982421875	0.999323844909668
4	1	1469.9541015625	1127.156982421875	0.9991412162780...
5	2	1469.9541015625	1127.156982421875	0.9991075396537...
6	3	1469.3389892578...	1127.027099609375	0.9992382526397...
7	4	1468.8505859375	1126.7528076171...	0.9991303086280...
8	5	1468.1878662109...	1126.7528076171...	0.9990606904029...
9	6	1467.821044921875	1126.61767578125	0.9991973042488...
10	7	1467.792236328125	1126.601318359375	0.9990272521972...
11	8	1467.2049560546...	1126.601318359375	0.999056875705719
12	9	1466.2740478515...	1126.480712890625	0.9988491535186...

Figure 11. Example Frame with the DLC Markers and Output. (A) Each body part of the mouse is automatically labelled with a marker to track its movement throughout the OFT session. The "tail base" coordinate (labelled in orange) is the body part used for free-roaming analysis. (B) Example of DLC output showing x and y coordinates of the "tail base" and the accuracy of the tracking estimate for each datapoint under the "likelihood" column.

Following DLC processing, PyRat, an open-source Python library, is used to convert coordinates from pixels to centimeters (cm).

Total distance traveled (in meters) is calculated by summing the Euclidian distances between consecutive time points. To assess anxiety-related behavior, the amount of time spent in the center of the open field is analyzed. The central area is defined as the region occupying 25% to 75% of the total field dimensions—corresponding to 12–28 cm in width and 15–35 cm in length.

8.3 Histology Data Analysis

To detect and quantify Methoxy-x04-labeled plaques in brain section images, a publicly available protocol based on ImageJ software (version 2.14.0/1.54f) is used. Each TIFF image captured at 10X magnification is calibrated and converted from pixels to millimeters.

A polygon is drawn to define the region of interest (ROI) for plaque analysis. The images are then converted to binary format, followed by the thresholding and watershed functions to separate plaques from the background. Then, the 'Analyze Particles' and 'Measure' functions are used to calculate the area of the ROI and quantify the total number of plaques within it. To enable standardized comparisons across samples, plaque counts are normalized to the ROI area, yielding plaque density in units of plaques/mm².

8.4 Statistical Analysis

To assess the statistical significance of observed differences among different experimental groups varying across two independent factors, a Two-Way ANOVA test is performed following data preprocessing to meet the assumption of normality. Normality is first assessed using the Shapiro-Wilk normality test on residuals. If the data are not normally distributed ($p < 0.01$), but high W – value (close to 1), a log transformation is applied to normalize the values before performing the ANOVA. If the data are normally distributed ($p > 0.01$), no further transformation is performed.

The Two-Way ANOVA reports the main effects of each factor (e.g., genotype and age) as well as their interaction effect. When significant differences are found from the ANOVA, Tukey's Honestly Significant Difference (Tukey HSD) post hoc test is used to identify which specific group comparisons are statistically significant.

Moreover, if no groups in the data are normally distributed ($p < 0.01$) and with low W – values, an alternative non-parametric Scheirer Ray Hare test is performed. Similar to Two – Way ANOVA this test also reports main effect of the factors and their interaction but uses ranks rather than raw data for analysis.

Lastly, a Student's t-test is performed on normally distributed data with one independent variable. The Student's t-test compares the means of two groups to determine if they are statistically different from each other.

II. Results

1. 5xFAD mice fail to mimic sustained weight loss and subsequent positive weight difference between young and old mice observed in human patients in the course of disease progression.

Eating abnormalities, weight loss, and reduced appetite are commonly observed in patients with AD, and these symptoms positively correlate with disease progression (Sergi et al., 2013; Shea et al., 2018). Interestingly, even in cases where appetite increases, weight loss often persists—suggesting underlying metabolic dysfunction. Given the complex interplay of factors such as weight, appetite, motor activity, and metabolic changes, identifying a single underlying cause for this weight loss remains challenging.

A previous study found that female 5xFAD mice exhibit weight loss and reduced food consumption at 6 months of age. However, contrary to expectations, this weight loss was not linked to motor activity, ruling it out as a primary contributing factor (Gendron et al., 2021).

To evaluate the robustness of these previously reported weight loss findings in 5xFAD mice, the body weight of each mouse was measured daily throughout the IBL training period. Compared to wild-type controls, 5xFAD mice showed less variability in weight within each age group across the training period (Fig. 12). Despite continuous water restriction during training, no significant weight changes were observed in any group; in fact, a slight weight increase over time was noted. Additionally, no significant weight loss was detected in either 5- or 7-month- old 5xFAD mice compared to controls.

To further investigate potential weight differences due to aging and disease progression, an additional analysis was conducted comparing younger and older mice across AD and wild-type genotypes. Initial body weights, measured on the first day of IBL training, were used to establish baseline differences across age and genotype groups. Based on existing literature documenting sustained weight loss with disease progression, it was reasonable to expect older (G2) AD mice to weigh less than age-matched controls.

Normality of residuals was assessed using the Shapiro-Wilk test. Results showed that weight distributions across age groups were normally distributed ($W = 0.95$, $p = 0.09$), whereas genotype-based distributions violated normality assumptions ($W = 0.91$, $p = 0.01$). To correct for this, weight data were log-transformed prior to analysis. A two-way ANOVA (Fig. 13) revealed a significant main effect of age on weight ($p < 0.01$), while genotype and the age-by- genotype interaction did not significantly influence body weight.

These findings suggest that the previously reported weight loss in 5xFAD mice was not replicated in this cohort. Moreover, the significantly higher body mass of G2 mice compared to G1 mice—regardless of genotype—suggests healthy weight maintenance, contradicting findings commonly reported in AD patients.

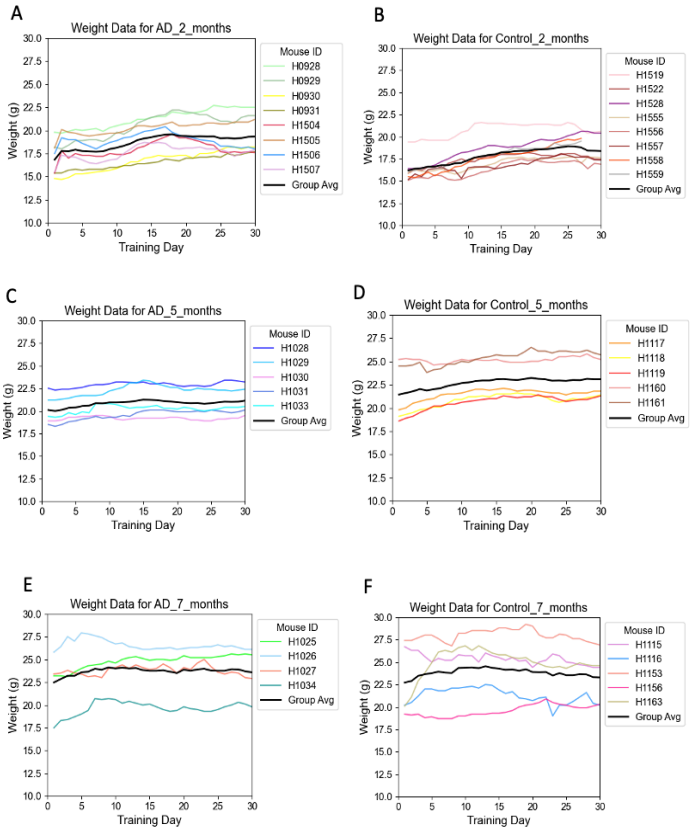


Figure 12. Body Weight Trajectories of Individual Mice Across Age Groups and Genotypes During Training. The body weight (in grams) of individual mice from each sub age group (2, 5, and 7 months) and genotype (AD and control) was tracked over 30 consecutive days of training. (A) and (B) show weight trajectories of 2-month-old AD and control mice, respectively. (C) and (D) depict weight data for 5-month-old AD and control mice. (E) and (F) present data for 7-month- old AD and control mice. Individual mice are represented by distinct colors, as indicated in the legends. The black line in each panel represents the group's average daily weight.

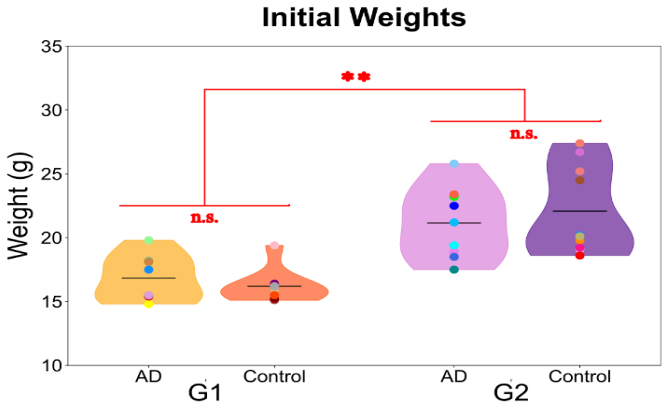


Figure 13. Initial Body Weight Comparison. The body weight (in grams) on the first day of task for each mouse across age and genotype groups. Two-ANOVA of the log transformed data, revealed a significant effect of age $F(1, 31) = 40.14$, $p < .001$, $\eta^2 = .56$ on the initial body weight. Post hoc test showed a significant difference between the G1 group ($M = 2.86$, $n = 16$) and G2 group ($M = 3.11$, $n = 18$), with a mean difference of -.25 ($SE = .04$), $t(1) = -6.46$, $p < .001$, Tukey's HSD. Initial weight was neither significantly affected by the genotype $F(1, 31) = .01$, $p = .90$, $\eta^2 < .01$ nor by the interaction between age and genotype $F(1, 31) = .84$, $p = .37$, $\eta^2 = .03$. Note: $^{*}p < .05$, $^{**}p < .01$.

2. Age but not genotype had a significant impact on learning in 5xFAD and control mice.

A plausible account for the inconsistent findings regarding cognitive deficits in AD mouse models is that many commonly used behavioral tests may be too simplistic, allowing mild impairments to be masked or compensated for by mice with greater cognitive reserve. If this is the case, more complex tasks that involve multiple cognitive domains may more reliably reveal deficits associated with disease progression in AD mouse models.

Another contributing factor to inconsistency is the influence of various confounding variables on cognitive test performance. For example, the Morris Water Maze (MWM) - a widely used assay for evaluating spatial memory in AD mice (see Introduction 6.2), lacks standardization and is sensitive to factors such as stress, anxiety, and motor impairment. This highlights the need for introducing standardized, well-controlled protocols to assess cognitive function more reliably.

The IBL task addresses these limitations of traditional cognitive assays by offering a highly complex and fully standardized platform. It requires mice to engage a wide range of cognitive domains, including sustained attention, motivation, sensory processing, long-term memory, and associative learning. Accordingly, mice with even mild cognitive deficit are unlikely to succeed in learning and performing the task. Indeed, a previous study found a sharp decline in learning ability and exploratory behavior with age in the IBL task, demonstrating its sensitivity to cognitive capacity (Hwang et al., 2023).

Furthermore, the IBL framework standardizes most aspects of the experiment—from surgical procedures and water restriction to training protocols, hardware, and software—minimizing variability and potential bias across studies. This makes the IBL task a powerful tool for robustly assessing complex cognitive function in AD mouse models.

Based on this rationale, 5xFAD and age-matched wild-type control mice were trained in the IBL task over the course of 30 days to assess their learning abilities and decision-making behavior. Mice were initially grouped by age at the start of training into three cohorts: 2, 5, and 7 months. However, as behavioral performance between the 5- and 7-month-old groups did not differ significantly, they were combined for analysis. Hereafter, G1 refers to 2-month-old mice, while G2 refers to the combined 5- to 7-month-old group. AD denotes 5xFAD mice, and control denotes wild-type mice.

As shown in the average learning curves (Fig. 14), younger mice (G1) learned the task more rapidly than older mice (G2) in both AD and control groups. On average, G1 mice reached greater than 80% correct response in less than 20 days of training, while G2 mice remained below 60% accuracy even after 30 days of training.

Surprisingly, genotype did not significantly impact learning performance in either age group: 5xFAD mice performed comparably to wild-type controls. This was unexpected, given 5xFAD mice show amyloid plaque accumulation as early as 2 months of age. These findings suggest that early amyloid pathology alone may not be sufficient to impair learning in this complex cognitive task.

To more precisely evaluate the influence of genotype and age on learning, task performance was analyzed across three distinct phases of training: early, middle, and late (Fig. 15; see Methods 8.1).

The Shapiro–Wilk normality test on residuals indicated that task

performance was normally distributed across all groups and training phases, with the exception of the age group during the initial phase (W = 0.91, p = 0.01). Taking into consideration high W-value (1 = very likely to be normally distributed), and the p-value being at the significance threshold, the data for this group is very likely to be normally distributed. Therefore, a Two-Way ANOVA was used to assess the effects of age and genotype on performance.

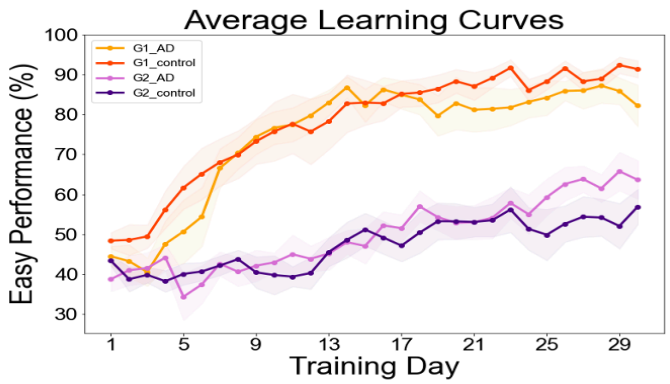


Figure 14. Average Learning Curves for G1 and G2 Groups. The y-axis represents the percentage of correct responses, and the x-axis indicates the corresponding training days. Bold lines represent the average learning curves for each group, with shaded regions representing the standard error of the mean. Group colors are as follows: G1 AD – yellow, G1 Control – orange, G2 AD – orchid, G2 Control – indigo.

During the initial phase (training days 1–5; Fig. 15A), age had a significant main effect on the performance (p = 0.02). No significant effects of genotype or the interaction between age and genotype were found. Overall, G1 mice outperformed G2 mice regardless of genotype. Within the G1 group, control mice performed better than AD mice, although this difference did not reach statistical significance.

In the middle phase (training days 16–20; Fig. 15B), age had a significant main effect on performance (p < 0.001), while genotype and the age × genotype interaction did not. The same pattern was observed during the last phase (training days 26–30; Fig. 15C), where age again significantly affected performance (p < 0.001), but genotype and interaction effects remained non-significant.

These quantitative comparisons reinforce the trends observed in the average learning curves: younger mice (G1) consistently outperformed older mice (G2), regardless of genotype.

Importantly, the absence of a significant genotype effect across all training phases suggests that 5xFAD mice were not impaired in learning the IBL task, despite extensive amyloid plaque accumulation. It is worth noting that the difference in control G1 mice compared to AD G1 mice is not observed later in the training reflecting on strong cognitive capacity of 5xFAD mice.

This finding challenges the hypothesis that inconsistent cognitive deficits reported in 5xFAD mice are due solely to the simplicity of behavioral assays. Instead, it supports the hypothesis that amyloid pathology alone may be insufficient to drive cognitive impairment, indicating a decoupling between amyloid burden and functional decline. This has important implications for understanding AD pathogenesis and for interpreting preclinical outcomes in amyloid-targeted models.

3. Exploratory behavior diminishes in relation to age but not genotype in 5xFAD mice.

Exploratory behavior—the tendency to investigate novel environments or stimuli—is disrupted early in the course of AD. For instance, patients with probable AD exhibit reduced exploratory eye movements, spending less time fixating on novel visual stimuli (Daffner et al., 1999). This reduction in exploratory behavior is thought to reflect broader symptoms of apathy and disengagement commonly observed in AD. Similarly, AD mouse models, including 5xFAD, have shown decreased exploratory tendencies, although the onset and nature of this decline remain unclear.

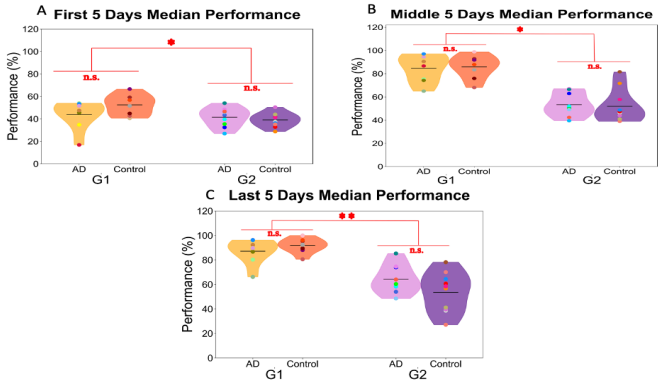


Figure 15. IBL Task Performance Across Three Training Periods. (A) During the first 5 days of training, task performance was significantly affected by age group, $F(1, 31) = 6.55$, $p = .02$, $\eta^2 = .16$. There was no significant main effect of genotype, $F(1, 31) = .76$, $p = .39$, $\eta^2 = .02$, and no significant interaction between age and genotype, $F(1, 31) = 3.00$, $p = .09$, $\eta^2 = .07$. Post hoc test revealed a significant difference in performance between the G1 group ($M = 48.14$, $n = 16$) and G2 group ($M = 40.23$, $n = 18$), with a mean difference of 7.91 ($SE = 3.20$), $t(1) = 2.47$, $p = .02$, Tukey's HSD. (B) During the middle 5 days, performance was significantly affected by age group, $F(1, 31) = 67.48$, $p < .001$, $\eta^2 = .69$. There was no significant main effect of genotype, $F(1, 31) = .001$, $p = .97$, $\eta^2 = .00$, and no significant interaction between age and genotype, $F(1, 31) = 0.10$, $p = .76$, $\eta^2 = .001$. Post hoc test revealed a significant difference in performance between the G1 group ($M = 85.26$, $n = 16$) and G2 group ($M = 52.66$, $n = 18$), with a mean difference of 32.59 ($SE = 3.85$), $t(1) = 8.47$, $p < .001$, Tukey's HSD. (C) During the final 5 days of training, performance remained significantly affected by age group, $F(1, 31) = 58.52$, $p < .001$, partial $\eta^2 = .62$. There was no significant main effect of genotype, $F(1, 31) = .87$, $p = .36$, partial $\eta^2 = .01$, and no significant interaction between age group and genotype, $F(1, 31) = 3.54$, $p = .07$, partial $\eta^2 = .04$. Post hoc test showed a significant difference between the G1 group ($M = 89.51$, $n = 16$) and G2 group ($M = 58.59$, $n = 18$), with a mean difference of 30.92 ($SE = 4.17$), $t(1) = 7.41$ $p < .001$, Tukey's HSD. Note: * $p < .05$, ** $p < .01$.

In this section, we investigated whether 5xFAD mice exhibit reduced exploratory behavior in a decision-making context. In the IBL task, wild-type mice typically make highly exploratory choices during the early phases of training—likely to gather information and identify strategies that maximize reward. This exploratory phase is considered a critical period for flexible learning.

To quantify exploratory behavior, we calculated the fraction of "switch" trials—those in which the choice differed from the previous trial—regardless of whether the previous choice was rewarded or not. This analysis focused on the first two days of training. The Shapiro–Wilk normality test confirmed that the distribution of switch trial fractions was normal. A Two-Way ANOVA was then conducted to assess the effects of genotype and age on early exploratory behavior.

The analysis revealed a significant main effect of age (p < 0.01), with older mice (G2) exhibiting a lower tendency to switch compared to younger mice (G1) (Fig. 16). However, no significant effect of genotype or age × genotype interaction was found. These results are consistent with prior findings of age-related declines in exploratory decision-making (Hwang et al., 2023), but do not support a specific impairment in exploration in 5xFAD mice.

A previous study also reported a positive correlation between early exploratory behavior and learning outcomes—suggesting that greater exploration facilitates more effective task acquisition. To examine this relationship in our dataset, we correlated each mouse's early exploratory behavior (fraction of switch trials during days 1–2)

with its task performance during training days 11–15, a period shown to be sensitive to individual differences in learning capacity. Although a positive trend was observed in both AD (Fig. 17A) and control groups (Fig. 17B), the correlations were not statistically significant ($p = 0.12$ and $p = 0.07$, respectively), likely due to limited sample size.

Taken together, these findings indicate that 5xFAD mice do not show impaired exploratory decision-making or learning compared to age-matched controls at either 2 or 5–7 months of age. This further supports the view that amyloid pathology alone may not be sufficient to produce measurable deficits in these complex cognitive functions.

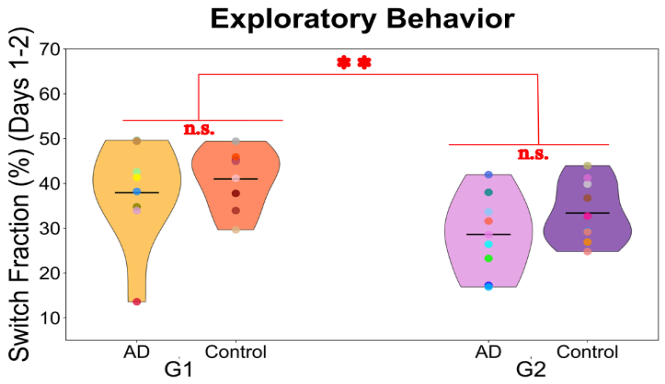


Figure 16. Exploratory behavior in day 1 and 2 of the IBL training. Exploration is significantly affected by age group $F(1,31) = 8.57$, $p < .01$, $\eta^2 = .21$. Post hoc test revealed a significant difference between G1 group ($M = 39.45$, $n = 16$) and G2 group ($M = 31.13$, $n = 18$), with a mean difference of 8.132 ($SE = 2.88$), $t(1) = 2.89$, $p < .01$, Tukey's HSD. No significant main effect for genotype, $F(1,31) = 1.93$, $p = .18$, $\eta^2 = .046$; and no significant interaction between age groups and genotypes, $F(1,31) = .09$, $p = .77$, $\eta^2 = .002$. Note: * $p < .05$, ** $p < .01$.

4. Age but not genotype affects the distance traveled in OFT in the first 5 minutes of the session.

Results from open field test (OFT) in 5xFAD mice have been highly variable and inconsistent across studies in the literature. Some suggest an increase in locomotor activity, while others decrease or no change. For example, certain studies have found that 12-month-old 5xFAD mice exhibit hyperactivity, traveling greater distances compared to age-matched controls, whereas other reports show no significant genotype effect on locomotor activity in 9-month-old mice. These conflicting findings leave it unclear how amyloid pathology influences locomotor behavior.

To address this issue, 5xFAD and wild-type control mice in the current study underwent the OFT following completion of the 30-day IBL training (Fig 18). Total distance traveled in the arena was used as a measure of locomotor activity. Two time points were observed to account for potential effect of habituation to the novel environment (see Methods 4). Due to mobility issues mouse H1117 was excluded from OFT analysis.

In the first 5 minutes, the Shapiro–Wilk test of residuals revealed that distance data were normally distributed across genotypes group ($W = 0.99$, $p = 0.96$), whereas distance across age groups was not normally distributed ($W = 0.89$, $p = 0.002$). Therefore, a log transformation was applied to meet the normality assumption prior to conducting a Two-Way ANOVA.

The analysis showed a significant main effect of age ($p < 0.01$), with older mice traveling less than younger mice (Fig. 19A). However, there were no significant effects of genotype or genotype × age interaction. These findings suggest that locomotor activity declines with age but is not significantly influenced by amyloid pathology in 5xFAD mice within the age range tested.

In the middle 5 minutes, the Shapiro–Wilk test of residuals revealed that distance data were normally distributed across ages ($W = 0.96$, $p = 0.19$) and genotypes ($W = 0.93$, $p = 0.04$). A Two- Way ANOVA was conducted to identify the main effects of age and genotype as well as their interaction. The analysis showed that none of the factors has a significant main effect and neither did their interaction (all p – values > 0.05) (Fig. 19B).

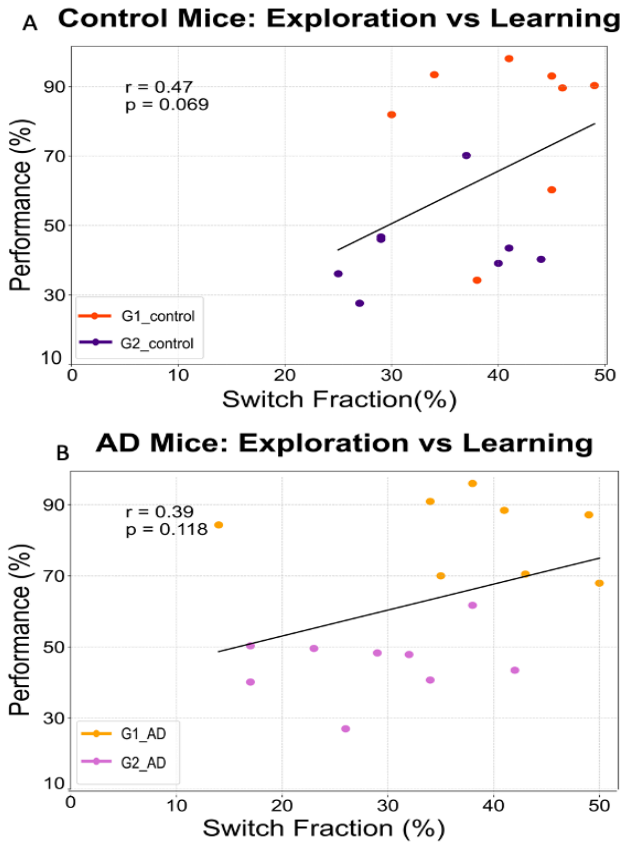


Figure 17. Correlation of Learning (Days 11-15) and Initial Exploration. (A) A positive but non- significant correlation was observed between learning and exploration in control mice across G1 and G2 age groups, $R_S = 0.47$, $p = 0.07$, Spearman's rank correlation ($n = 34$). (B) A positive but non-significant correlation was observed between learning and exploration in AD mice across G1 and G2 age groups, $R_S = 0.39$, $p = 0.12$, Spearman's rank correlation ($n = 34$).

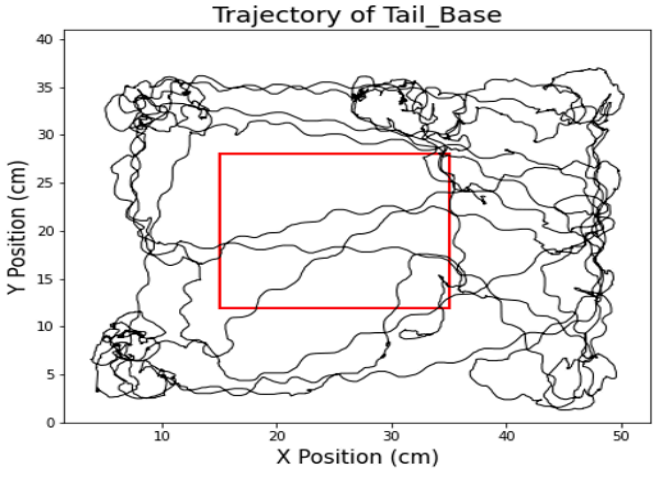


Figure 18. Tail Base Trajectory in OFT of a Mouse. Plot representing the path

traveled by the mouse during the first five minutes of the OFT. The x- and y-axes are scaled in centimeters to represent the actual movement range of the mouse.

Taken together, these results indicate that G1 mice traveled a greater distance at the beginning of the session compared to G2 mice, potentially reflecting increased anxiety manifested as hyperlocomotion. As the session progressed, however, no differences in locomotor activity were observed between the groups, suggesting the influence of habituation. This pattern raises the question of whether the initially larger distance traveled is related to heightened exploratory behavior (Sil et al., 2022) which has been previously associated with younger mice (see Fig. 16). Notably, this cohort did not exhibit any AD - related effects on motor activity in either age group, contributing to the ongoing discussion of whether amyloidosis alone is sufficient to produce non-cognitive symptoms in 5xFAD mice.

5. Time spent in the center of the arena of open field linked with anxiety phenotype does not change with age or AD progression.

Anxiety-like behaviors are among the most prevalent and debilitating neuropsychiatric symptoms observed in patients with AD, significantly impairing quality of life and daily functioning. Therefore, it is essential to investigate anxiety phenotypes in transgenic mouse models of AD to better understand the underlying mechanisms and identify potential therapeutic targets. Despite extensive research in 5xFAD, findings in this area remain inconsistent. Some studies report an increase in anxiety-like behaviors with disease progression, while others observe no significant changes—or even a decrease (Padua et al., 2024). This variability underscores the need for standardized behavioral protocols and careful interpretation of results within the context of both disease stage and model-specific characteristics. A conventional method for assessing anxiety-like behavior in mice is the Open Field Test (OFT), which measures the amount of time an animal spends in the center of an open arena. This metric is based on the natural tendency of mice, as prey animals, to avoid open and exposed spaces. Increased time spent in the periphery is typically interpreted as an anxiety-like response, whereas more time spent in the center suggests reduced anxiety. Thus, reluctance to explore the center of the arena is commonly used as an indirect measure of heightened anxiety. A recent study showed no significant difference in time spent in the center between 5xFAD and control mice (Zhu & Liu, 2025).

In this research, anxiety in G1 and G2 mice across both genotypes was evaluated measuring the fraction of time they spent in the center of the field in the first and middle 5 minutes of the OFT session (see Method 8.2). In the first 5 minutes, the Shapiro–Wilk test of residuals revealed that time spent in the center of the field was not normally distributed across ages ($W = 0.67$, $p < 0.01$) and genotypes ($W = 0.70$, $p < 0.01$). As neither W- value not p-value were close to being normally distributed, Scheirer Ray Hare test - a non-parametric Two-Way ANOVA was performed to evaluate the main effect of factors and their interaction. The analysis showed no significant main effect of age ($p = 0.84$), genotype ($p = 0.42$) or genotype × age interaction ($p = 0.17$). (Fig. 20A)

In the middle 5 minutes, the Shapiro–Wilk test of residuals revealed that time spent in the center of the field was not normally distributed across ages ($W = 0.87$, $p < 0.01$) and genotypes ($W = 0.81$, $p < 0.01$). For the same reason as described earlier, Scheirer Ray Hare test was performed to measure the main effect of factors and their interaction on the anxiety phenotype. The analysis showed no significant main effect of age ($p = 0.10$), genotype ($p = 0.69$) or genotype × age interaction ($p = 0.94$) (Fig. 20B).

These findings suggest that 5xFAD mice do not express levels of anxiety that significantly deviate from wildtype control mice supporting a segment of the previously provided research. Additionally, anxiety in G1 mice does not show difference compared to G2 mice. This enhances the debate about the effectiveness of the model in studying behavioral deficits consistently presented in human patients.

6. Plaque deposition is observed throughout the AD progression starting at 2 months with no pronounced differences across age groups.

Plaque deposition in 5xFAD mice differs from that observed in humans. In these mice, extracellular plaque deposits initially form in the subiculum, deep cortical layers, and frontal cortex, and with age, they progressively spread to the cortex, subiculum, and hippocampus. Alongside the accumulation of β-amyloid plaques, neuroinflammation caused by reactive glial cells also emerges, showing an age-dependent progression and a similar pattern of regional spread (Pádua et al., 2024). To verify consistency with this established timeline, we examined brains from 2-month-old naïve 5xFAD mice (i.e., not exposed to behavioral testing; see Fig. 21). As expected, early Aβ aggregates were detected in the subiculum, supporting previous findings.

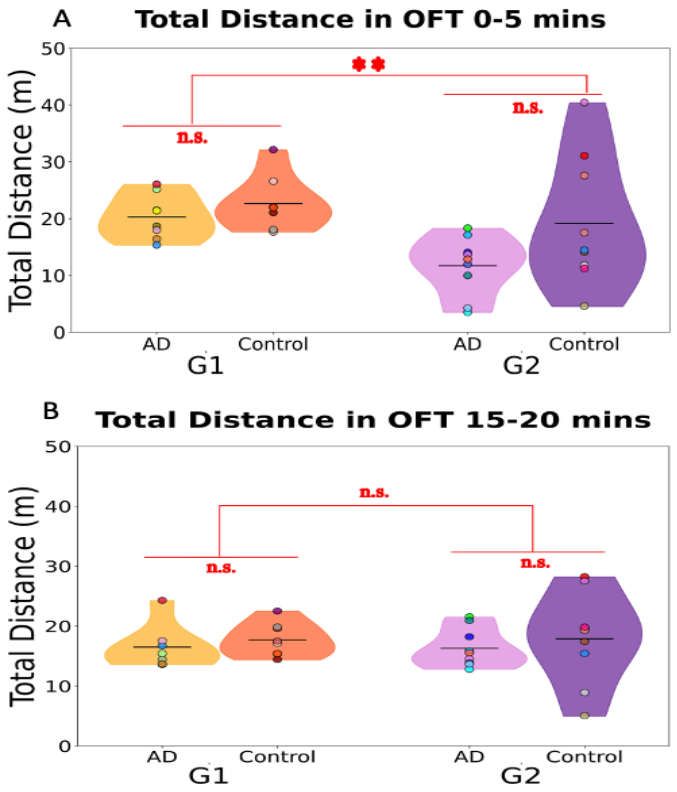


Figure 19. Distance Travelled in the First and Middle 5 Minutes of the OFT. (A) During the first 5 minutes of OFT the distance travelled was significantly affected by age group, $F(1, 30) = 9.13$, $p < .01$, $\eta^2 = .21$. There was no significant main effect of genotype, $F(1, 30) = 3.23$, $p = .08$, $\eta^2 = .07$, and no significant interaction between age and genotype, $F(1, 30) = 1.07$, $p = .31$, $\eta^2 = .03$. Post hoc test revealed a significant difference in performance between the G1 group ($M = 3.10$, $n = 16$) and G2 group ($M = 2.65$, $n = 17$), with a mean difference of .45 ($SE = .15$), $t(1) = 2.92$, $p < .01$, Tukey's HSD. (B) During the middle 5 minutes of OFT the distance travelled there was no significant main effect of age $F(1, 29) = .00$, $p = 1.00$, $\eta^2 = .00$, genotype, $F(1, 29) = .70$, $p = .41$, $\eta^2 = .02$, and no significant interaction between age and genotype, $F(1, 29) = .01$, $p = .91$, $\eta^2 = .00$. Note: * $p < .05$, ** $p < .01$.

Given the distinct differences in plaque deposition between humans and 5xFAD mice, it is critical to examine the subiculum's role in cognition. In humans, the subiculum is one of the earliest regions to exhibit volumetric reduction in AD, and its atrophy has been closely associated with cognitive decline (Baset & Huang, 2024). Functionally, the subiculum acts as a central hub, integrating input from hippocampal areas and relaying output to various cortical and subcortical regions. Together with the CA1 region of the hippocampus, the subiculum has been strongly implicated in verbal and episodic memory. Episodic memory, in turn, plays a key role in decision-

making processes, such as temporal discounting—where individuals are more likely to reject rewards that are delayed in time (Moscovitch et al., 2016). While relatively less research has focused on the specific functional role of the subiculum in rodents, existing evidence suggests that deficits in associative learning begin to emerge around 5 months of age in AD mouse models (Pádua et al., 2024). This onset likely corresponds with the progression of amyloid plaque deposition and the disruption of projections to synaptically connected regions, such as the prefrontal cortex and entorhinal cortex. However, due to the limited number of studies directly addressing subicular function in this context, it remains challenging to draw definitive conclusions. This gap highlights the importance of assessing learning abilities across different age groups in mouse models of AD to better understand the temporal relationship between neuropathology and cognitive decline.

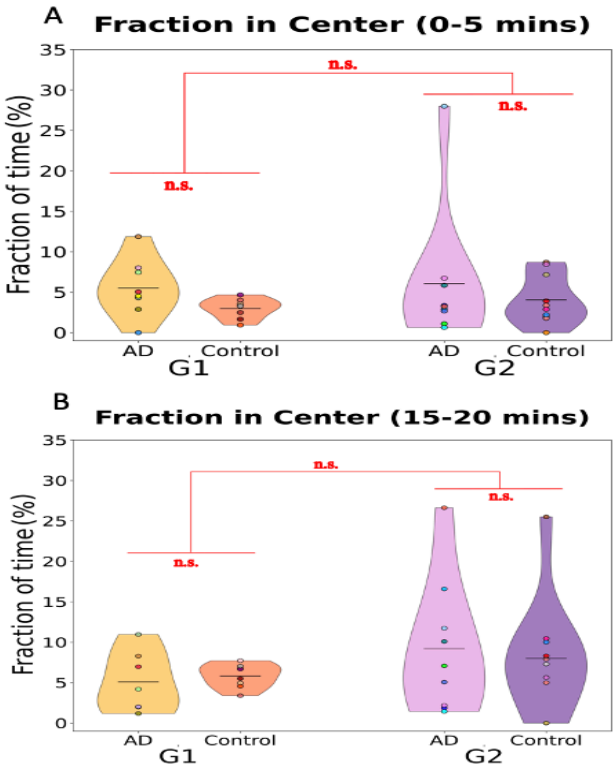


Figure 20. Fraction of Time Spent in the Center of the Arena in the First and Middle 5 Minutes of OFT. (A) During the first 5 minutes of OFT the time spent in the center was not significantly affected by age group, H(1, 1) = 0.04, p < .84, genotype, H(1, 1) = .66, p = .42, and no significant interaction between age and genotype, H (1, 1) = 1.89, p = .17. (B) During the middle 5 minutes of OFT the time spent in the center was not significantly affected by age group, H (1, 1) = 2.65, p< .10, genotype, H (1, 1) = .16, p = .69, and no significant interaction between age and genotype, H (1, 1) = 0.01, p = .94. Note: * p <.05, ** p <.01.

The hippocampus (HPF) is one of the most prominently affected brain regions in AD. Unlike the subiculum, its role in learning and memory has been extensively studied, and it is well- established that the hippocampus is critical for memory formation, particularly episodic and spatial memory (Eichenbaum, 2004). In AD patients, hippocampal atrophy is one of the earliest and most consistent biomarkers, closely correlating with cognitive decline, especially in the early stages of the disease (Braak & Braak, 1991). Furthermore, there is a well-documented age-related increase in Aβ plaque deposition in the hippocampus, which is strongly associated with synaptic dysfunction and progressive learning and memory deficits (Mormino et al., 2009). Girard et al. (2014) suggests that 5xFAD mice starting at 4 months exhibit declarative-like memory deficit failing to link correct cue-reward-association even in perfectly mastered task.

These findings highlight the hippocampus's direct and possibly indirect role in the deterioration of cognitive functions in AD. We will further assess the relationship between cognitive and pathological changes as a result of AD in 5xFAD mice.

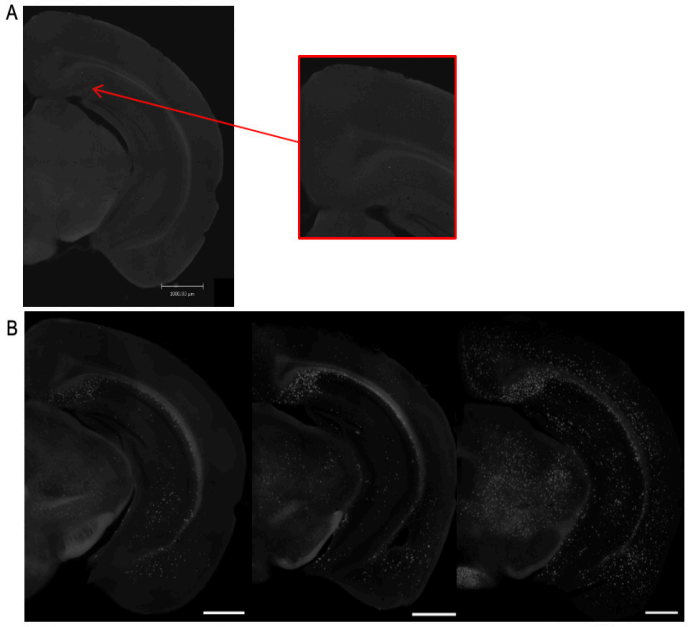


Figure 21. Plaque Aggregation in 5xFAD Mice at Different Age. (A) Right side subiculum of the coronal brain slice in 5xFAD mouse at the age of 2 months training naïve. (B) Right side coronal brain slice in 5xFAD mouse 3 – 4 (G1), 7, 9 (G2) months of age, respectively (at the time of tissue collection, post training).

Plaque density in subiculum was calculated for mice across G1 and G2 groups. A Shapiro – Wilk test of residuals reveled that plaque density was normally distributed (W = 0.93, p = 0.26). An independent student T-test was conducted to compare the differences between groups. The analysis showed no significant differences in the plaque deposition of G1 group compared to G2 (p = 0.53) (Fig. 22A). The same analysis was conducted for plaque density in hippocampus. A Shapiro–Wilk test of residuals revealed that plaque distribution was normally distributed across ages (W = 0.94, p = 0.42). An independent student T-test was conducted to compare the differences between groups. The analysis showed no significant differences in the plaque deposition of G1 group compared to G2 (p = 0.22) (Fig. 22B). The findings indicate that the subiculum is more densely packed with plaques compared to the hippocampus, suggesting a regional pattern in plaque deposition and potentially reflecting the sequential involvement of these structures in plaque formation. Although no statistically significant differences were observed in plaque density between G1 and G2 groups in either region, it is possible that plaques in G2 are more densely clustered while maintaining a similar overall quantity to G1, which may reflect differences in plaque compaction rather than total load. This regional disparity, with greater plaque density in the subiculum, supports the hypothesis that it may be more vulnerable or involved earlier in the pathological progression than the hippocampus.

7. Plaque density in SUB and HPF did not impact cognitive abilities of the mice.

Given the significant differences in cognitive performance between the G1 and G2 age groups, plaque density may serve as a contributing factor to the observed decline. To examine this relationship, the middle five training days (see Methods) were correlated with plaque density in two brain regions of interest for each mouse. To provide greater insight, mice were analyzed separately by age group at the time of brain extraction, G1 included mice aged 3–4 months while G2 consisted of mice aged 7 and 9 months. Although negative trends were observed across all age groups in both the HPF (Fig. 23A) and SUB (Fig. 23B), these correlations did not reach

statistical significance—except for the 9-month-old group in SUB, where a significant association between plaque burden and cognitive performance was identified. These findings suggest a potential link between plaque accumulation and cognitive decline that may not be solely age-dependent. However, due to the correlational nature of the analysis, it remains unclear whether increased plaque density directly contributes to cognitive deficits, or if cognitively impaired mice are more susceptible to plaque aggregation.

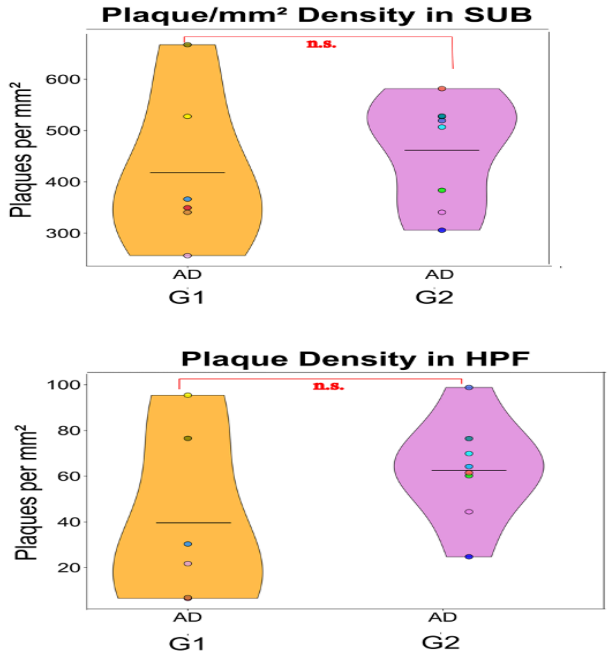


Figure 22. Plaque density in SUB and HPF (plaque/mm^2). Independent student T-test showed no significant difference between plaque density in SUB in G1 and G2 age group AD mice, T(8.35)= -0.61, p = 0.57, η^2=0.09. Independent student T-test showed no significant difference between plaque density in HPF in G1 and G2 age group AD mice, T(7.51)= -1.34, p = 0.22, η^2=0.27.

III. Discussion

In the current study, the 5xFAD mouse model, widely used for investigating pathological and behavioral changes associated with AD, was utilized to address several gaps in knowledge regarding the timeline of pathology development and its relationship to behavioral changes. The primary goal was to investigate whether the pathological changes in plaque accumulation observed in these mice correspond to behavioral changes, particularly cognitive and non- cognitive performance.

Weight Analysis

Initially, weight changes were analyzed to explore whether the weight loss observed in human AD patients, which often correlates with disease progression, would also be evident in the 5xFAD mice. Interestingly, no such trend was observed. Both age groups showed consistent weight across genotypes (Fig. 12), with G2 mice exhibiting significantly higher body weight compared to G1 (Fig. 13). This finding aligns with an extensive analysis of 5xFAD mice by Forner et al. (2021), who reported no weight differences across genotypes until around 8 months of age. The absence of weight loss in the 5xFAD model contrasts with the common clinical feature of weight decline in human AD patients, suggesting that this mouse model may not fully replicate all aspects of the disease, particularly those associated with metabolic disturbances.

Cognitive Behavior

Behavioral changes, both cognitive and non-cognitive, were explored to better understand the impact of pathology on function. Cognitive performance, assessed using the IBL task, revealed a slight decrease in task performance in the G2 group compared to G1, though both AD and control mice displayed similar levels of performance (Fig. 14A, B, C). These results are consistent with previous findings in this lab, reinforcing the sensitivity of the IBL task to detect cognitive differences (Hwang et al., 2023). However, no significant genotype-related differences were observed, raising the question of whether cognition and decision making as one of the domains of cognition is affected in AD pathology.

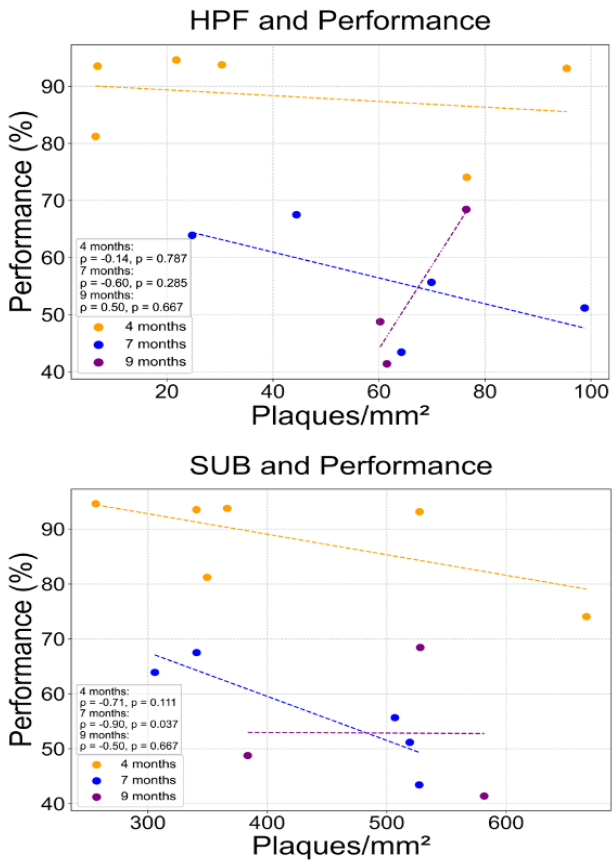


Figure 23. Correlation of Learning (Days 16-20) and Plaque Density in HPF and SUB in Each Age Group. (A) No significant correlation was observed between learning and plaque density in HPF across age groups, specifically 4 months R_S = -.14, p = .79, 7 months R_S = -.60, p = .29, 9 months R_S = .50, p = .67, Spearman's rank correlation (total n = 14). (B) No significant correlation was observed between learning and plaque density in SUB across age groups, namely 4 months R_S =- .71, p = 0.11, 9 months R_S =- .50, p = 0.67. Only 7 months mice showed negative significant correlation between plaque deposition in subiculum and their task performance R_S =- .90, p = 0.04, Spearman's rank correlation (total n = 14).

Non-Cognitive Behavior

While we anticipated alterations in exploratory behavior and anxiety levels based on the pathological changes, the results were different. Contrary to expectations, the 5xFAD mice, regardless of age group, did not show altered exploratory behavior, a finding that contrasts with the decreased exploration seen in human AD patients even in early stages of the disease. However, G1 mice demonstrated increased exploratory behavior, which is in line with earlier findings suggesting that younger mice are more motivated to explore and engage in exploratory behavior more than in older mice (Hwang et al., 2023). Despite this, no definitive conclusions were drawn regarding differences between AD and control mice across age groups, which could be attributed to the small sample size of this cohort.

OFT Analysis

The Open Field Test (OFT), commonly used to assess locomotion and anxiety, yielded some unexpected findings. No genotype-related differences were observed in the distance traveled during the first 5 minutes, contradicting earlier studies that reported reduced locomotion in

5xFAD mice compared to controls at 4 to 6 months of age (Poon et al., 2023). However, a significant reduction in distance traveled across age groups was detected, potentially reflecting age-related declines in exploration or motor abilities (Fig. 19). Interestingly, during the middle 5 minutes of the test, neither genotype nor age-related differences were observed, suggesting that habituation to the environment may have influenced the behavioral outcomes.

When anxiety was assessed based on time spent in the center of the arena, no significant differences were found at any time point. This finding contributes to the ongoing debate in the literature, as studies have variously reported increased, decreased, or unchanged anxiety levels in 5xFAD mice (Padua et al., 2024; Zhu & Liu, 2025) (Fig. 20A, B). A key strength of the current study is its ability to evaluate the effects of habituation by comparing distinct time windows within a single continuous OFT session, offering more nuanced insights into the behavior of 5xFAD mice.

In human patients, non-cognitive symptoms often precede cognitive decline and serve as strong predictors for disease progression (Ismail et al., 2016). The inconsistency in non- cognitive behavioral outcomes in this mouse model raises an important question: to what extent do 5xFAD mice faithfully replicate this early domain of AD?

Plaque Accumulation Analysis

Histological analysis of plaque accumulation revealed results consistent with previous studies, demonstrating rapid plaque deposition beginning as early as 2 months of age. Initial accumulation was evident in the SUB, followed by progressive spreading to additional brain regions with age (Fig. 21A). This stage-like progression, particularly the early involvement of the SUB, parallels patterns observed in other AD models and supports the notion that this region may be among the first to exhibit pathological changes. Notably, plaque burden did not significantly differ across age groups (Fig. 22A,B), in line with prior reports (Forner et al., 2021). Although statistical comparisons did not show significance, aggressive age-dependent plaque spreading was clearly visible in tissue images (Fig. 21B). This observed plaque burden suggests that other pathological mechanisms—such as tau aggregation or neuroinflammation—may play a more prominent role in cognitive decline during later disease stages.

While correlations between plaque burden and cognitive performance were not statistically significant across most age groups, a consistent negative trend was observed, indicating a potential link that warrants further investigation with a larger sample size. It is important to consider that this trend may reflect age-related factors rather than plaque accumulation per se. However, this interpretation is complicated by findings in the 7-month-old AD group, where a significant negative correlation was identified in the SUB, suggesting that increased plaque burden is associated with poorer task performance. In contrast, the 9-month-old group exhibited a non-significant positive correlation between HPF plaque burden and task performance, while the negative relationship in the SUB persisted. These results suggest two possible interpretations: first, that age may exert a stronger influence on task performance than plaque deposition alone; second, that specific brain regions, such as the SUB, may differentially contribute to cognitive outcomes.

Despite these patterns, it remains unclear whether increased plaque accumulation directly causes cognitive decline, or whether mice with reduced cognitive performance are more prone to plaque deposition. Alternatively, these results may highlight a limitation of the IBL task itself, which may not be sensitive to detect the specific cognitive deficits associated with the 5xFAD genotype—particularly in domains such as episodic and spatial memory.

These findings raise broader concerns about the translational relevance of the 5xFAD model. If it fails to robustly replicate both cognitive and non-cognitive features of AD, its utility as a comprehensive preclinical model may be limited. In humans, substantial subicular atrophy and hippocampal dysfunction are recognized as early indicators of AD, underscoring their importance in the cognitive domain. Accordingly, it would be reasonable to expect impaired performance in 5xFAD mice due to disruptions in cue–reward association learning, mirroring deficits observed in human patients.

Conclusion and Implications

Overall, the findings from this study provide valuable insights into the timeline of pathology development and the behavioral consequences in the 5xFAD model of AD. While we observed early plaque deposition and some behavioral changes in line with previous studies, the lack of significant behavioral changes in the 5xFAD model challenges us to reconsider how we measure cognitive decline and anxiety in AD. These findings suggest that the 5xFAD model may not fully recapitulate the range of clinical symptoms observed in human AD, particularly in the early stages of the disease. Future studies should explore additional behavioral tasks, larger sample sizes, and longer longitudinal analyses to better understand the connection between early pathology and cognitive decline in AD. Additionally, plaque quantification in the cortex across the observed ages might give a stronger insight about the relationship of plaque burden and cognition.

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