Characterizing Behavioral Phenotypes in the 5xFAD Alzheimer's Disease Mouse Model Using Computer Vision-based Monitoring

By

ALEX B. VON ECKARTSBERG

HISTORY, BA, MIAMI UNIVERSITY, OHIO, 2011

Thesis

Submitted in partial fulfillment of the requirements for the Degree of Master of Science in the Graduate Program of Biotechnology at Brown University

PROVIDENCE, RHODE ISLAND

MAY 2024

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Date_____ Signature: _____

Dr. JUSTIN FALLON, Advisor

Date_____ Signature: _____

Dr. Yong Jong, Reader

Date_____ Signature: _____

Dr. Alexander Fleischmann, Reader

Approved by the Graduate Council

Date_____ Signature: _____

Dr. Thomas Lewis, Dean of the Graduate School

Acknowledgments

Thank you to Dr. Justin Fallon, my advisor during this project. I had no idea what was in store for me when I came to Brown, or what I would conduct my research on. Dr. Fallon and I had such great interactions from the beginning, and he entrusted me with the perfect project during my time here. For that, I will always be grateful. A special thank you for also being so considerate of my life as a full-time student as I also work full-time and raise my two young boys. His understanding of my situation inspired me, and I am indebted to him.

Additionally, our lab manager, Beth McKechnie, could not have been more supportive. Beth, you were always keeping me honest during our experiments and coaching me through the nuances of the lab. I could not have done any of this without you, and I know that after 27 years of dedication to the lab, there are so many more who feel the same way. Thank you for all you did for me, and the other students who remember you so fondly. Happy retirement, and stay in touch!

I also want to thank Lauren Fish, a recent PhD graduate of the Fallon Lab. Lauren embodies all the critical qualities not just of a fantastic lab member, but of a selfless leader. She continues to support me in my research, even after she has departed Brown, and I commend her outright dedication to her teammates and myself. Thanks, Lauren!

I would also like to thank my committee members; Dr. Yong Jong and Dr. Alexander Fleischmann. Dr. Jong inspired me to pursue the business side of biotechnology and begin my Executive MBA next year at Georgetown University. Dr. Fleischmann's class expanded my threshold of critical and creative thinking in how I viewed research - something I will use well after I graduate from Brown. He also was incredibly supportive during the birth of our second son, so thank you, Dr. Fleischmann! Also, thank you to our program director, Dr. Jacquelyn Schell, who helped steer us in the right direction from the start.

Lastly, I want to say how much I cherish my family. Atlas, you have grown into such a sweet young man while we have lived in Providence, and you have been excited for the adventure from day one. Otto, we were so happy to welcome you into the world last year, your smile is contagious and I can't wait to see the young man you grow into. Anita, my life, words can not show how much you mean to me. Thank you for migrating away from our home with our family further North than you ever wanted to - I won't ever forget it. Amo te, meu coração.

Table of Contents

Table of Figures

Abstract

Abstract of Characterizing Behavioral Phenotypes Using Computer Vision-based Monitoring in an Alzheimer's Disease Mouse Model, by Alex von Eckartsberg, ScM, Brown University, May 2024

By 2017, only four approved drugs to treat symptoms of AD existed, while a resounding 146 drug candidates had failed to be approved by the FDA. Today, this theme remains, with only a single disease modifying therapy (DMT) in lecanumab (Leqembi ®) gaining approval while showing underwhelming results in slowing the progression of the disease. While characterizing the neuropathological changes that occur in testing these therapies on mice and other animals is critical for preclinical development and ultimately FDA approval, behavioral assessments that capture changes in motor function over time are limited. Historically, neuromotor behavioral testing in mouse models includes open field analysis, grip strength analysis, rotarod, and wire hang, among others. While these traditional-style tests provide useful information, they can be prone to human error and inconsistencies. In this study, we use a common Alzheimer's Disease (AD) mouse model and assess motor dysfunction and behavior using an unbiased, automated, AI-powered recording model entitled Automated Continuous Behavioral Monitoring (ACBM). The 5xFAD transgenic mouse is most conducive to testing motor dysfunction due to excessive amyloid plaque build-up. At 8 months, a novel behavioral phenotype of "early activity onset" was identified showing 5xFAD mice becoming highly active approximately one hour earlier than WT mice during early morning hours. Additionally, phenotypes confirming historical trends in 5xFAD behavior were identified in both Walk, Hang, and Sniff behaviors showing significant increases hyperactivity, along with decreased body composition compared to the WT mice. Importantly, our novel comprehensive

analysis of groom activity in the 5xFAD mouse is only preceded by one other study monitoring the behavior for just 5 minutes during open field testing (Ullah et al., 2020). Our data suggest that not only does ACBM confirm previous behavior summarized by traditional behavior testing, but that it can deliver consistent monitoring while identifying potentially new behaviors or deficits powered by computer vision that would otherwise not be known. Our lab continues to utilize ACBM to monitor additional ages of 5xFAD mice and others to form a comprehensive dataset with the ultimate goal of testing new DMTs/anti-amyloids, such as lecanumab, to observe any behavioral changes.

Chapter 1: Introduction

1.1 | Alzheimer's Disease

In 1906, Alois Alzheimer made an announcement at the 37th South-West German Psychiatrist conference in Tübingen, Germany regarding recent findings from a female patient he had been monitoring for over five years. He noted to his colleagues "A peculiar severe disease process of the cerebral cortex" and conveyed histology consisting of plaques and neurofibrillary tangles (Hippius, et al., 2003). Alzheimer's Disease (AD) is a neurodegenerative disease that remains one of the most elusive and costly affecting society today while offering no cure. Characterized by a progressive decline in cognition, onset of the disease can vary over time and is influenced both by genetics and an individual's environment and lifestyle choices. The spectrum of severity within AD varies from early stage preclinical AD, to mild cognitive impairment (MCI), followed by mild, moderate, and severe AD. Preclinical AD is characterized by amyloid pathology in patients without any signs or symptoms, while MCI indicates cognitive impairment is present but not severe enough to be characterized as AD (van der Flier et al., 2023). While there are a plethora of pathological changes that contribute to AD, it is clinically defined by the presence of intercellular excess depositions of β-amyloid (Aβ) peptides (plaques) along with intracellular neurofibrillary tangles (NFT) of hyperphosphorylated tau protein. Progression of the disease occurs over 20-30 years, beginning with the rise in amyloid plaques years before deposits of tau proteins are formed (Bateman et al., 2012; Jansen et al., 2015). Once AD has been diagnosed, however, the brain is no longer able to be rescued and cognitive decline will continue. Symptoms

of AD patients include memory loss, judgment, and loss of physical abilities such as walking, sitting, and eventually swallowing (Alzheimer's Association, n.d.).

Currently, there are 6.7 million people in the US with AD. That number is projected to climb to 13.8 million people in 2050 (Alzheimer's & Dementia: The Journal of the Alzheimer's Association, 2020). Astoundingly, the U.S. is spending $3.7 billion each year on AD research since the National Alzheimer's Project Act (NAPA) was passed and signed into law in 2011 (Skaria, 2022). In 2022, the financial burden of AD was assessed to be $321bn with future costs in 2050 amounting to over $1 trillion. While the financial backing for AD is strong, as mentioned, the success of clinical trials has been grim including the current selection of therapies on market (RePORT: National Institute of Health, n.d.).

1.2 | Pathology Challenges of Alzheimer's Disease

AD has shown to be increasingly difficult to treat and develop therapeutics that target the underlying neuropathology. There are several contributing factors to this complexity. One such factor is the location of the disease. The brain is sequestered from the rest of the blood-brain barrier (BBB), an extremely selective set of vascular connections that limit the passage of molecules into the central nervous system (CNS) (Hardy et al., 1991). Made up of multiple cell types, including endothelial cells (ECs) and tight junctions among others, their function is to keep out toxins and other harmful substances from entering the brain area. This limitation prevents virtually all macromolecular therapeutics except for the limited uptake (0.1-0.2% of injected doses) of circulating antibodies (Kouhi et al., 2021), and 98% of all small molecule drugs from entering the

brain to treat the disease (Pandit et al., 2020; Banks, 2016), stemming from tight junctions formed by endothelial cells which only allow for passive diffusion of lipid-soluble drugs under 400-600 Da (Wu et al., 2023). Likely the biggest challenge surrounding AD is that no one single target exists capable of defeating the disease. This couldn't be more apparent than it is now that after three decades of mainly targeting amyloid plaques, patients have only one DMT option that shows moderate effectiveness in slowing the progression of the disease. Due to this lack of treatment successes, the pharmaceutical industry has already started to broaden their scope of targets outside of amyloid plaque into tau pathology, inflammation factors, epigenetic factors, and others in various stages of the drug development pipeline (Cummings et al., 2023).

1.3 | Genetic Risk Factors of Alzheimer's Disease

Another factor contributing towards the difficulty of treatments are the genetics associated with AD. In a larger genome-wide association study (GWAS) meant to identify new genetic variants involved in contributing to the disease, found the number of AD-associated risk alleles was more than 40 (Fig. 1) (Jansen et al., 2019; Scheltens et al., 2021). Early onset AD (EOAD), sometimes referred to as familial AD (FAD), occurs in patients before the age of 65 years and accounts for 1-5% of all AD cases (Goldman et al., 2011). The three causative gene mutations and duplications resulting in autosomal dominant EOAD are amyloid precursor protein (APP), Presenilin-1 (PSEN1), and PSEN2 accounting for an estimated 5.3 per 100,000 persons being at risk, or less than 1% of all AD patients (Campion et al., 1999; Lanoiselée et al., 2017; Dong et al., 2022). While the most frequent cause of AD from these variants is PSEN1, inheritance of any will

ultimately lead to the pathogenesis of AD (Xiao et al., 2021). Late onset AD (LOAD), sometimes referred to as sporadic AD (SAD), occurs in patients after the age of 65 years and is the more complex form of the disease accounting for 95% of all AD cases (Eid et al., 2019). While multiple genes serve as risk factors for the sporadic version of the disease, the strongest risk factor remains mutations within the ε4 allele of the Apolipoprotein E gene (APOE), translating to the APOE4 isoform (Scheltens et al., 2021). One allele of the APOE4 mutation increases the risk of AD by three times, while two copies of the allele increase the risk by roughly 12 times (Liu et al. 2013; Jia et al. 2020).

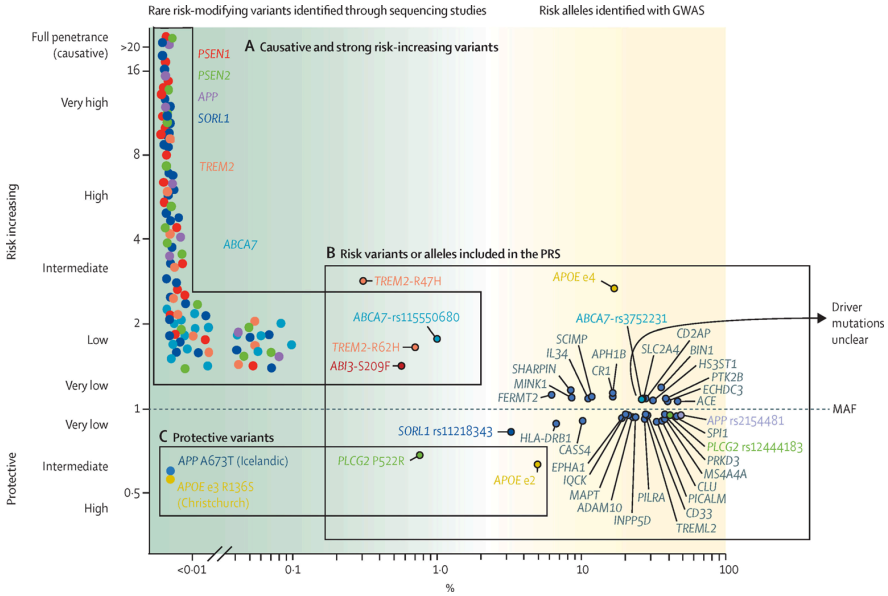


Figure 1.1: **The Genetic Factor**. Depiction of the many variant alleles based on risk level that contribute to why the disease is so difficult to defeat. A) **Causative or strong risk variants.** PSEN1, PSEN2, and APP cause autosomal dominant AD in some patients as early as 40 years of age, which is more rare (minor allele frequency (MAF) <0.01%). B) **Risk variants.** Genome-wide association studies (GWAS) show much more common variants (MAF>1%) representing risk alleles such as APOE4. C) Protective variants (MAF<0.01%) are also rare likely to provide a resistance against disease-associated risk factors of cognitive decline. From (Scheltens et al., 2021)

1.4 | Diagnosis of the Disease

Diagnosis for AD remains a difficult and somewhat ambiguous task for clinicians today. There is no single test that exists to effectively identify the disease, nor is there a single result that provides conclusive evidence. In parallel, there is also no single target or "kill switch" for the disease, a reality that has been painfully apparent over the past 30 years with failed attempts to create a therapy that can halt the disease entirely. Today's standard for diagnosing AD includes collecting medical history, cognitive screening, and physical examination by a physician. From this initial assessment, often there can be determined if a patient demonstrates dementia (experiencing cognitive impairment that impairs daily activities), mild cognitive impairment (MCI) (experiencing cognitive impairment in one or more domains, but maintains global cognitive function), or subjective cognitive decline (complaints of cognitive issues without impairment on any cognitive tests). Etiologic diagnosis requires neural examinations, basic laboratory tests, MRI or CT scans of the brain by a specialist. Additional testing such as PET brain imaging using biomarkers and analysis of cerebrospinal fluid (CSF) would also likely take place (Teipel et al., 2022). The U.S. National Institute on Aging and Alzheimer Association supports diagnosis criteria of preclinical AD with the positive presence of biomarkers, such as CSF or PET imaging, and the absence of cognitive impairment (Sperling et al., 2011). (descriptions here of levels of biomarkers). Critical to these assessments, is the understanding that AD should be viewed as a continuum, one that typically develops over a period of 15 to 25 years with dementia itself being the final results of an extended AD pathological presence (Scheltens et al., 2021).

More recently, GWAS have provided useful insights into the genetic etiology of AD by identifying risk loci - as mentioned in Section 1.3 - but are typically limited to detecting low frequency/low risk variants. However, second-generation methods including whole genome sequencing (WGS) and whole exome sequencing (WES) have discovered rare and ultra-rare AD-associated variants such as TREM2, ABCA7, PLCG2, and ABI3, which hold critical roles in microglial efferocytosis in AD (Khani et al., 2022). While these genotyping technologies are robust in nature, the cost of generating datasets that are sufficiently powered continues to be the most challenging aspect with these techniques (Andrews et al., 2023).

Additionally, the field of proteomics has shown promise by identifying several plasma protein biomarkers that could have the potential to detect AD up to 15 years in advance. In a recent study examining data from the UK Biobank, researchers identified GFAP, NEFL, GDF15 and LTBP2 proteins to be associated most with all-cause dementia (ACD), Alzheimer's disease (AD) and vascular dementia (VaD), with GFAP being noted as an optimal biomarker for dementia more than 10 years before the diagnosis (Guo et al., 2024). Just as new technology is being leveraged to gain insights in diagnosis of AD, advanced computer vision and artificial intelligence (AI) techniques can assist in an automated way to characterize the behavioral phenotypes of animal mouse models that could prove invaluable during the testing of potential drug therapeutics.

1.5 | Mouse Models of Human Disease

Characterizing the genetics of a disease can provide invaluable insights into its pathology. Researchers can replicate this genetic data in animal disease models, such as mice, which can model specific disease-associated mutations. Mice are more cost effective than many other animal models, they breed extremely well, and around 90% of the mouse and human genome can be divided into conserved syntenic regions (Breschi et al., 2017). Mouse models can also allow for the new discovery of novel disease phenotypes, signaling pathways and additional genetic functions related to the disease. One of the more important functions of these mouse models lies in the ability to test early phase drugs during the drug discovery process. Specifically, relevant disease mouse models provide essential preclinical data showing metabolism and absorption, efficacy, and general safety of these early-stage medicines.

Alzheimer's disease mouse models are crucial tools in understanding the pathology and progression of the disease. These models are engineered to exhibit characteristics similar to those seen in human Alzheimer's patients, such as the accumulation of amyloid-beta plaques and tau tangles, synaptic dysfunction, and cognitive decline. Researchers have developed various types of mouse models, including transgenic mice that overexpress mutant forms of genes associated with familial Alzheimer's disease, as well as knock-in and knock-out models targeting specific genes involved in disease pathways. More recently, inflammation-based mouse models have also been incorporated in AD research with the notion of microglia playing an important role in the pathogenesis. Depending on the aim of research, there are mice that display Aβ deposition such as the 5xFAD and APP23 mice, and tauopathy mouse models such as PS19 and JNPL3 (Yokoyama et

al., 2022). In addition to these pathogenic phenotypes defined based on the model, locomotor and behavioral testing such as open field analysis and grip strength provide valuable insight into functional qualities of these diseased models.

1.6 | Familial Alzheimer's Disease Mouse Model: 5xFAD

The 5xFAD mouse line is a genetically modified strain developed to mimic aspects of familial Alzheimer's disease (FAD). It carries five mutations associated with familial forms of Alzheimer's disease, including three mutations in the human amyloid precursor protein (APP) gene (Swedish (K670N/M671L), Florida (I716V), and London (V717I)) and two mutations in the human presenilin 1 (PSEN1) gene (M146L and L286V). These mutations accelerate the production and aggregation of amyloid-β (Aβ) peptides, leading to the formation of amyloid plaques, a hallmark feature of Alzheimer's disease (Oakley et al., 2006). PSEN1 is a protein involved in the γ-secretase complex, which plays a crucial role in the processing of APP - a transmembrane protein that undergoes proteolytic processing to produce Aβ peptides. Cleavage of APP by β-secretase and γ-secretase enzymes generates Aβ peptides, which can aggregate and form amyloid plaques in the brain.

1.7 | Traditional Locomotor & Behavioral Testing

Traditional locomotor and behavioral testing in mouse models evaluate neurological traits and other events such as memory, socialization, age of onset, and longitudinal progression amongst other traits (Uslu, 2021). Functional testing to capture the timing of disease progression and

identifying early and novel phenotypes are extremely valuable for researchers so that once therapeutic interventions are introduced, a measured change in behavior is clearly defined.

Common methods for this type of testing include grip strength analysis, which is a specific measurement of the force required to pull before the mouse releases the narrow bar it places its grip. One limitation of this method is often the lack of motivation mice show in hanging onto the bar (Brooks et al., 2009). The wire hang test measures endurance and muscle coordination, where the mice are suspended on a wire or wire grid. The time between the mouse's placement on the wire to when they let go is captured (Hoffman et al., 2016). Similar to the grip strength testing, mice are often unmotivated to hang onto the wire at all so this will not accurately measure their capacity to hang until fatigued (Brooks et al., 2009). Another common behavior test is the open field analysis which measures emotionality and motor parameters such as ambulation, latency, and rearing. This evaluation consists of a wall-enclosed area with sufficient height to prevent the mouse from escaping, and should elicit a feeling of openness in the center of the maze. There is often the addition of objects which adds to measuring the ability of interaction between the mouse and the object (Seibenhener et al., 2015). Often, mice who have higher levels of anxiety will spend more time near the edges of the maze walls as opposed to the center, and usually cover less distance traveled compared to their less-anxious counterparts. While these techniques have been standard practice in characterizing diseased mouse model behaviors, they also inherently include varying levels of bias and require human intervention throughout the testing period.

1.8 | Automated Continuous Behavioral Monitoring (ACBM)

Neurodegenerative disease-related behaviors, particularly in the genesis, are often too subtle to be detected by traditional behavioral animal model testing. Additionally, human intervention and bias could inadvertently skew results of these tests. For these reasons, our lab has collaborated with the Thomas Serre Lab to apply Automated Continuous Behavioral Monitoring (ACBM) to identify mouse behavior and motor phenotypes. ACBM is a computer vision-based model using deep learning to characterize and quantify specific behaviors through automated, sensitive, and objective means with zero human intervention. Due to the sensitive and robust nature of ACBM, it has the capacity to readily identify these phenotypes across mouse lines. Importantly, ACBM also offers a structured and consistent means to evaluate these behaviors - something that eludes the research community today with the use of traditional behavioral testing. Previously, use of ACBM has identified early and novel behavioral phenotypes in the TDP-$43^{Q331K/Q331K}$ amyotrophic lateral sclerosis (ALS) mouse model that were not detected using traditional behavioral analysis (White et al., 2018).

Originally, ACBM was developed based on the organization of motion processing in the dorsal stream of the primary visual cortex where low-level motion features are processed first, followed by more complex and invariant features (Jhuang et al., 2010; White et al., 2018). Data collected for this processing takes place in the form of individually housed mice recordings at 30 frames per second for 5 days of collection, yielding approximately $1.3x10^7$ frames per mouse per session. Using a semi-supervised learning approach with previously labeled behavioral data, the AI-based deep learning model is trained to classify 9 distinct behaviors based on individual frames

of the recording; eat, eat-by-hand (EBH), drink, walk, rear, hang, sniff, rest, and groom. Once the data is processed and average can be taken across each day and genotype. Experimental output provides the number of seconds that each mouse spends with respect to each behavior during the circadian hour. Differences among the time spent for each of the 9 behaviors can also be calculated between genotypes.

Compared to traditional behavioral testing, ACBM offers a reproducible, automated, and unbiased approach to capturing the neurobehavioral features of diseased mouse models. Such unbiased approaches would benefit the drug development pipeline which consistently displays a huge disparity between clinical testing in the early phases of research, from clinical outcomes. Testing options such as ACBM remove the data collection and analysis limitation, with the ability to collect and process enormous amounts of data that would otherwise take humans thousands of hours. While this study is focused on the 5xFAD mouse model, analysis across mouse lines in AD can be classified and compared, including traversing into other neurodegenerative disease mouse models. We also propose that ACBM could readily be used to test new therapies in the drug development pipeline against the common therapeutic targets of anti-amyloid plaques and hyperphosphorylated tau in an automated, repeatable, and standardized format to characterize behavioral traits.

Chapter 2: Materials and Methods

2.1 | Mice

Two different genotypes of mice were used in this study. There were a total of 16 mice classified during the recording sessions; 8 wildtype (WT) mice used in this study were C57BL/6J ordered from JAX laboratory, along with 8 5xFAD hemizygous mice shipped from MMRRC via JAX laboratory.

All procedures for our study were approved by the Institutional Animal Care and Use Committee (IACUC) at Brown University and done in accordance with institutional guidelines for animal care. Upon arrival at Brown from JAX, both WT and 5xFAD mice were kept in group housing with a max of four per cage (i.e. two cages of four mice each for both cohorts). Once ACBM recording began, mice were transferred to single housed ACBM cages and remained in single housing for the duration of their time in animal care. The animal care facility was kept at a standard 12 hour light/dark cycle running from 8am to 8pm.

2.2 | Weight Analysis

This cohort listed in the "2.1 Mice" section above was weighed at the beginning and end of a five day recording cycle during 8 months of age.

2.3 | Food Monitoring/Recording

Prior to placement of both WT and 5xFAD mice in recording cages, initial weights of food provided were taken. Post-recording of the food in each respective cage was also weighed to capture the food displaced during this time.

2.4 | ACBM Video Recording Setup

Each mouse in the cohort was housed individually for the duration of the five day ACBM recording session. Each cage was monitored with a Firefly MV 0.3 MP Mono FireWire 1394a (Micron MT9V022) at 30 frames per second with cameras connected to a workstation with Ubuntu 14.04 using a Firewire card to connect to all cameras. A total of 16 mice (8 WT and 8 5xFAD) were recorded at an 8 month time point.

2.5 | ACBM deep learning assessment of mouse behavior

Our group's approach for ACBM was to leverage the spatio-temporal processing abilities of a Two-Stream Inflated 3D ConvNet (I3D) for automated labeling of animal behaviors from laboratory recordings (Carreira et al., 2017). To provide ground-truth labels based on human assessment, we used in-house software to manually annotate 9 behaviors described as follows:

1. Drink: Mouse drinks from a water spout
2. Eat: Mouse reaches for the feeder
3. Eat-by-hand (EBH): Mouse uses forepaws to eat while sitting on haunches
4. Groom: Mouse uses front paws to clean the face or uses one of the legs to clean the body
5. Hang: Mouse hangs from the ceiling of the cage
6. Rear: Mouse stands on hind legs with forepaws raised off the ground
7. Walk: Mouse moves using all four legs
8. Rest: Mouse stays still for an extended period of time
9. Sniff: Mouse sniffs the floor bed or the air while remaining static

Since obtaining annotations from scratch is extremely laborious, and is often inaccurate across annotators, we used a previous system developed in-house (Jhuang et al., 2010) to generate initial behavior labels which were then vetted by trained annotators. The initial training dataset for the I3D consisted of ~2.1M frames from 115 independent videos (an aggregated playtime of 19.76 hours). Further rounds of bootstrapping, a process in which we iteratively refined the I3D's performance on new videos, were performed as additional training steps for the I3D module. In total, ~83K frames sampled from 106 videos (an aggregated playtime of 0.77 hours) were selectively chosen, manually annotated, and injected back into the training procedure. Eventually, the best-performing I3D model was able to achieve a balanced classification accuracy of 77% over the 9 behaviors as described above on a held-out test dataset that consisted of ~21K frames over 8 videos (an aggregated playtime of 0.2 hours).

In a bid to further improve the system's performance, we explicitly modeled action class-specific temporal relationships by training a Bi-directional Long Short Term Memory (Bi-LSTM) recurrent neural network on the feature outputs of the I3D. In essence, the Bi-LSTM replaces the final classification layer of the I3D. Overall, this model reached a balanced accuracy of 81%, reaching the inter-human agreement ceiling.

2.6 | Statistical Modeling and Analysis of ACBM Data

Previous work has analyzed ACBM data at each hour using Repeated Measure ANOVA. While this method is valid for comparing two samples, it has limited utility for comparing complex data sets where the outcomes are bounded or do not follow a Normal distribution, and it may

sacrifice efficiency. Here we collaborated with Patrick Gravelle in the Gutman Lab at Brown to use a zero-inflated hierarchical generalized Dirichlet multinomial (ZIHGDM) regression model with cyclic splines to assess statistical differences in behavior times across the mouse genotypes. We let behavior times be the outcomes of interest and incorporate into the model's predictors mouse characteristics (e.g. age, gender), the knock-in type (mouse genotype), random effects for the day of recording, random effects for the mouse, cyclic splines on the hour of observation, and an interaction between the cyclic spline and knock-in type. Using this model, we estimate the difference between the genotypes for each of the behaviors to identify behavioral phenotypes. This model allows us to reduce animal-animal and day-day variability as well as gain additional information across behaviors. We implement the models within the Bayesian framework with appropriate prior distributions and utilize Markov Chain Monte Carlo algorithms to estimate all parameters. We perform posterior predictive checks to examine the fit of the model. Subsequently, we generate Bayesian confidence intervals, called credible intervals, from the posterior distribution of the parameters to assess mean differences between knock-in types in mouse behavior. Intervals that do not include 0 are considered significant.

Chapter 3: Results

3.1 | 5xFAD Hemizygous mice are runted and show increased weight gain during recording

To better understand metabolic-like factors of these mice through each recording period, all 5xFAD (hemi) and WT mice were weighed before and after recording sessions took place at 8 months of age (Fig. 3.1a). The 5xFAD mice weighed an average of 32.1g through both weights, compared to WT mice average weight of 35.1g. 5xFAD mice were found to weigh 8.92% less than

the WT mice after the recording session. Interestingly, the percent difference between 5xFAD and WT mice before the recording was 16.9%, showing a greater increase in weight gain in 5xFAD mice during the recording session (Fig. 3.1b). 5xFAD mice accounted for 18g of weight gain while WT mice accounted for -2g, across the five day recording period.

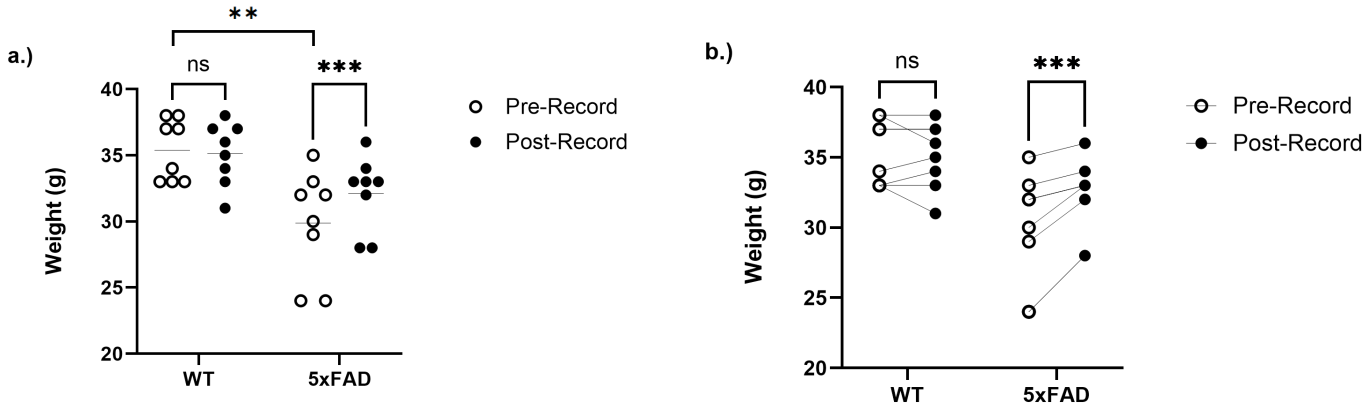


Figure 3.1: 5xFAD mice are runted and have a greater increase in weight gain during recording (a.) 5xFAD mice weigh less than WT mice at 8 months of age, both before and after recording showing runted factor in these 5xFAD mice **(b.)** Individual weight trajectories show all 5xFAD mice gained weight during recording compared to WT mice who were stagnant in weight gain, with several mice actually losing weight during the recording session. (2-way ANOVA 5xFAD p value = 0.0004, 2-way ANOVA WT v 5xFAD Pre-record p value = 0.0018)

3.2 | 5xFAD mouse cages showed less food displacement from food hopper compared to WT

In continuing our approach to capture any evidence of metabolic changes between 5xFAD mice, we also weighed food before and after the 5 days of ACBM recording (Fig. 3.2). While the recordings were not conducted in metabolic cages, this data is still useful in identifying correlations between ACBM-captured behaviors such as “Eat” and “EBH”. It’s also important to note that while these food weights can be useful, these food weights represent food displaced from the food

hopper and either eaten by the mouse or simply fallen into the cage floor. At 8 months, we observed 21.0% less food displaced from the 5xFAD mouse cages compared to WT. While this is consistent with data showing 5xFAD mice are runted compared to WT mice overall, it does not coincide with the data showing that 5xFAD mice gained over one-and-a-half more times the amount of weight, on average, as WT mice during the 5 day recording period.

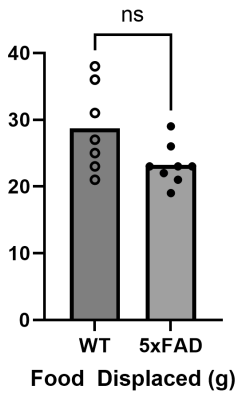


Figure 3.2: 5xFAD mice show less food displaced during 8 month recording session 5xFAD mice show less food displaced compared to WT mice, but interestingly show no difference in Eat or EBH compared to WT mice using ACBM. Many scenarios could contribute to food displacement, such as the mouse taking the food and eating only a small portion before leaving on the cage floor, or the food simply falling down from the cage. (t-test p-values WT vs. 5xFAD: p=0.05)

3.3 | ACBM provides a robust, unbiased, and automated disease phenotype assessment

Using ACBM, we were able to utilize a robust and automated method of characterizing multiple behavioral phenotypes simultaneously over a 5 day period without constant human oversight. Upon collecting data at 8 months of age, we could identify the number of seconds per hour each mouse in the cohort performed eat, EBH, drink, hang, rear, walk, sniff, rest, and groom (Supp. Fig. 1). Overall, the data showed typical behavior such as an increase in activity in the dark hours and less activity and higher levels of rest during the daytime hours. We saw no significant differences in Drink, Eat, EBH, Rear, and Rest, outside of the "early activity onset" in the early hours (Section 3.5). In the next sections, we discuss data collected during 8 months of age surrounding motor activity, "early activity onset", and alterations in grooming.

3.4 | ACBM reveals 5xFAD mice with an increase in motor activity/excitability

Since we observed 5xFAD mice during this period as runted but gaining a significantly more amount of weight during the recording compared to WTs, we were curious if ACBM would identify any correlation to the levels of motor activity. Data from this period of collection show a significant increase in 5xFAD motor behaviors Walk, Hang, and Sniff, with night hours showing overall greater activity levels compared to WT mice (Fig. 3.3). The greatest levels of activity between these behaviors is between 0500-0730 (5am-7am) and around 1600 (8pm), both just before and after the mice have slept, respectively.

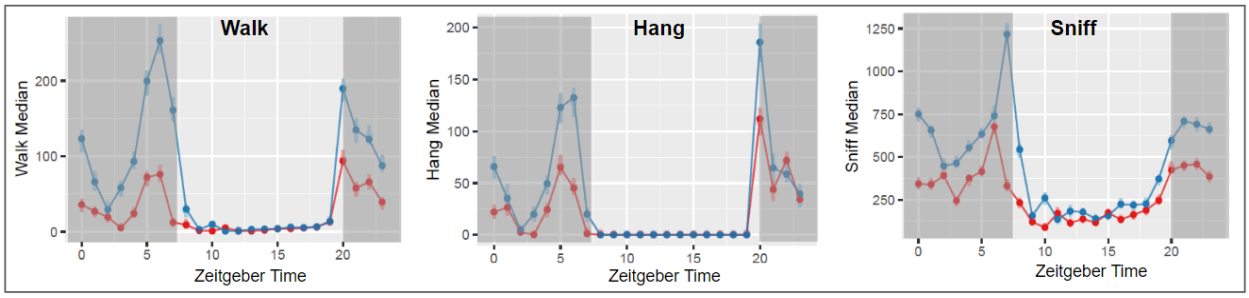


Figure 3.3: 5xFAD mice Walk, Hang, and Sniff levels are significantly greater than WT at 8 months The 5xFAD mice Walk and Sniff at the highest levels just before resting between 0500-0730, while the highest level of Hang behavior is seen just after waking up from daylight hours around 2000. Data shows similar levels of activity during the daylight hours when mice are mostly inactive. Blue = 5xFAD Red = WT

3.5 | ACBM identifies 5xFAD mice with a decrease in grooming

While we noticed an increase in activity by 5xFAD mice during active hours of the day, we also wanted to understand the psychological behaviors such as grooming were performed during the same timeframe. Interestingly, opposite of our findings related to increased activity of the

5xFAD, Groom levels were almost exclusively greater across all 24 hours compared to 5xFAD mice (Fig. 3.4). The greatest levels of grooming - both 5xFAD and WT - occurred just before dark hours around 1930 (7:30pm).

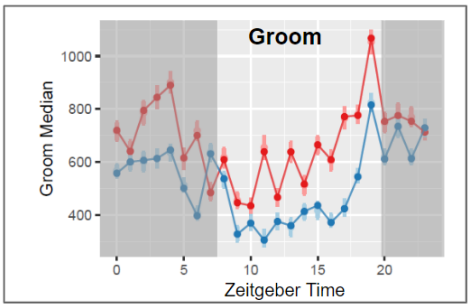


Figure 3.4: 5xFAD mice show a generalized decrease in grooming activity throughout the 24 hour period at 8 months The overall Groom levels are greatest during the most active hours of the day, with a strong increase as the daylight hours come to an end between 1900-2000. The most active grooming time is uniformly around 1930 between both groups. Blue = 5xFAD Red = WT

3.6 | ACBM displays 5xFAD mice showing "early activity onset" phenotype

There was a novel phenotype captured by ACBM related to an increase in activity across multiple behaviors in the early morning hours (0200-0300 or 2am-3am), just before the standard wake up time around 0330 (Supp. Fig. 1). The sudden uptick in activity can be observed clearly in 5xFAD mice conducting Drink, Eat, EBH, Hand, Rear, Rest, Sniff, and Walk behaviors compared to WT mice.

Chapter 4: Discussion and Future Directions

4.1 | Overview

This study has characterized the 5xFAD transgenic mouse model of AD using ACBM at the 8 month time point. While there have been many traditional methods testing non-cognitive behavioral and psychological symptoms (BPSD) of mice, these experiments inherently contain bias and a high level of human supervision and interaction which could skew the results. Our team set

out to characterize commonly used 5xFAD mice with a non-traditional, automated, and unbiased method in ACBM. 5xFAD mice overexpress five familial mutations leading to robust amyloid neuropathology that begin in the brain at 2-4 months of age. This accumulation leads to microgliosis and inflammation in addition to synaptic and neuronal loss (Forner, et al., 2021). Importantly, our ACBM data confirms some previously identified phenotypes of 5xFAD mice seen in traditional testing such as hyperactivity, while showing some striking new developments surrounding "early activity onset" and a novel profile of grooming behavior. Our data supports the validity and utility of ACBM in characterizing AD mice, given similar results to traditional testing and possible evidence into new phenotypes we have not yet seen based on this single age. Admittedly, more ages within the model should be assessed in order to determine if these phenotypes are transient or worsen over time.

4.2 | 5xFAD mice show hyperactivity along with a novel "early activity onset"

Our data shows a significant increase in 5xFAD activity in the Walk, Hang, and Sniff behaviors along an overall greater activity level compared to WT mice. An intriguing addition to this change in motor behavior was a consistent early active onset occurring between the hours of 0200-0300 in 5xFAD, approximately one hour prior to any activity assumed by WT mice under the same conditions (Fig. 3.3). Past non-automated studies have shown variability in activity levels of 5xFAD mice, showing reduced locomotor activity in 5xFAD mice between 12 and 15 months (O'Leary et al., 2018 & 2018; Schneider et al., 2014), hyperactivity at similar ages (Flanigan et al., 2014); Paesler et al., 2015), along with no difference in motor activity (Jawhar, et al., 2012; Yang et

al., 2017). In human AD patients, non-cognitive behavioral and psychological symptoms (BPSD) including hyperactivity, disinhibition, impulsive behavior, apathy, anxiety, and depression are common (Wang et al., 2022; Fernández et al., 2010; Alves et al., 2017; Kales et al., 2019). While the mechanisms of these behaviors remain elusive, hyperactivity can typically be seen in prodromal AD patients and (Kimura et al., 2019; Gallagher et al., 2017; Kim et al., 2021) may be associated with Aβ-derived neuronal over-activation (Harris et al., 2010; Lee et al., 2020; Balthazar et al., 2014). Modulation of the 5xFAD mouse model results underlines the importance of an automated and unbiased format like ACBM that can assess multiple days of behavior instead of brief segments of behavior as seen in traditional studies.

4.3 | 5xFAD mice depict a decreased level of self-care/grooming

During this 8 month age, 5xFAD mice had a significantly lower level of grooming activity compared to WT mice through every hour observed outside of 0630-0700. This seemingly novel neglect for grooming is the only study where 5xFAD mice have an extensive period of observed and recorded levels of grooming activity. Previous results observing the behavior of 5xFAD mice at 5-6 months of age saw an increase in grooming, however, this study measured only a single time point and observed the animals grooming behavior for 5 minutes during an open field test (Ullah et al., 2020). While receiving data in such a comprehensive format spanning over five days, additional ages of 5xFAD mice in this behavior will be useful to assess and provide additional analysis to properly capture a lasting phenotype.

Additionally, levels of grooming in mice can provide an important value to translational psychiatry and be useful for capturing the neural circuits involved in increasingly complex sequential patterns of action (Kalueff et al., 2016). This connection between deficits in grooming leading to motor impairments in AD mice has also been seen in Parkinson's Disease mice (Paumier et al., 2013).

In AD patients, neuropsychiatric symptoms (NPS) such as apathy-like behaviors are common. In mice, decreased grooming can either account for motor deficits or a lack of motivation/apathy-like behavior (O'Leary et al., 2024). Apathy-like behaviors such as reduced nest-building and impaired self-grooming, along with poor coat conditions, have a positive association with Aβ neuropathology severity in 5xFAD mice (Keszycki et al., 2023). Given that in our ACBM data of 5xFAD mice showing hyperactive behavior, we can assume that a lack of motivation or apathy-like behavior is the most plausible explanation for such a difference in the level of grooming observed by these 5xFAD mice.

4.4 | 5xFAD show stark increase in weight compared to WT mice

5xFAD mice displayed a noticeable increase in weight during the five day duration of ACBM recording at 8 months of age. While the 5xFAD mice are runted compared to the WT mice (Fig. 3.1a), they showed a 7.26% increase in average weight compared to just a 0.71% average increase in weight gain by the WT mice (Fig. 3.1b). Previous traditional behavioral testing have shown a reduction in body weight of 5xFAD mice during 9-10 months (O'Leary, 2018), while others have shown that 5xFAD males and females with mostly the same average weight at WT

between months 4 and 8, but beginning in month 12 through month 18 weighing significantly less than their WT counterparts (Forner et al., 2021).

During this same period, 5xFAD mouse cages had 21% less food displaced from their hoppers compared to WT mice, yet ACBM shows no significant change in Eat, EBH, or even Drink behaviors. Importantly, since this was the first recording of this cohort, both 5xFAD and WT mice were transitioning from group housing during shipment to Brown University, into a stressor environment of single housing during these recording periods. Due to male territorial and hormonal tendencies, these mice remained isolated throughout the recording lifecycle. While both genotypes had similar housing transitions, one potential reason for such an increase in weight could have been due to a dominant mouse in the group setting that could have stymied the appetites of the other mice. However, from Figure 3.1b, it's apparent that all mice have a similar increase in weight, and we do not see at least one mouse staying the same weight or even losing weight compared to the others which might have provided credence to having a more dominant mouse within the group.

In addition to the changes of food and weight, metabolic changes and vascular dysfunction is said to precede the progression of AD. Such neuropathological markers as Aβ accumulation and neuronal loss constitute these abnormalities in 5xFAD mice, along with reports of abnormal capillaries and attenuation of the blood brian barrier (BBB) that include a reduction in junctions and permeability of the vessels in younger aged mice (2-4 months) (Kook et al., 2012; Giannoni et al., 2016; Ahn et al., 2018; Jullienne et al., 2023). Numerous studies using 18F-fluorodeoxyglucose (FDG) uptake in PET, a marker particularly useful for determining mild cognitive impairment, to

measure cerebral glucose metabolism in 5xFAD mice have shown both an increase in FDG uptake in the hippocampus in 5xFAD mice aged 8-12 months (Choi et al., 2021) and ages 6 and 12 months (Jullienne et al., 2023), while others showed reduced FDG uptake in females at 7 months (Bouter et al., 2021) and males at 7 and 12 months (Franke et al., 2020). Disparities in these results likely relate to PET analysis techniques used by various labs, or potentially the nuanced WT strains of mice being used. Ultimately, these findings suggest that Aβ accumulation may be associated with a decrease in vascular delivery and an increase in metabolic demand which likely contributes to cognitive deficits (Jullienne et al., 2023). Importantly, such disruptions in human AD patients including cerebral hypoperfusion have also been reported to contribute to hypometabolism (Levin et al., 2021). Given the variable results seen in past studies and a lack of significant differences in Eat, EBH, and Drink phenotypes in this study, a more comprehensive assessment of the 5xFAD mouse using ACBM at ages before and after the 8 month period should provide a more enriched data for this topic.

4.5 | ACBM use as a therapeutic drug discovery method

One of the objectives of our research was to provide a consistent, repeatable, and unbiased behavioral characterization method to test prospective AD therapeutics in the drug discovery pipeline, including anti-amyloid monoclonal antibodies targeting Aβ plaques, along with vaccines and anti-tau antibodies targeting the tau protein. Offering alternative methods to validate efficacy in some disease modifying therapies (DMTs) is critical, mainly due to their only being one approved DMT on the market today in lecanumab (Leqembi®). It's worth noting that by 2017,

there were 146 drug candidates that failed approval to treat AD, with only four therapies actually receiving FDA approval - all of which treated the symptoms of the disease. In 2018, there were 26 drugs in Phase III clinical trials for AD, 17 of which were DMTs, and none were ultimately approved by the FDA mostly due to not producing a clinical benefit to the patient or the drugs not reaching their primary endpoint (Cummings et al., 2018). Needless to say, the journey to providing meaningful clinical outcomes in AD has proven difficult.

There has also been a noticeable increase in therapeutics the industry has seen in the last seven years, with a total of 105 AD drugs under evaluation in 2017. As of January 1, 2023, there were 187 clinical trials (Phase I-III) evaluating 141 drugs for AD categorized under either DMTs (biologics or small molecule), cognitive enhancer, or neuropsychiatric symptoms (Cummings et al., 2023). There has been steady growth in the number of evaluated drugs in the pipeline (Fig. 4.1), and while most Phase III drugs today are focused on targeting anti-amyloid beta plaques, more DMTs in Phase II and I are taking alternative approaches focusing more on Inflammation/Immunotherapy like targeting tau. This increase in therapeutics underlines the importance of establishing automated techniques such as ACBM within the drug development pipeline.

After three decades of chasing the amyloid hypothesis and utilizing the same traditional behavioral assessments, it's time the research community embraced artificial intelligence (AI) and machine learning (ML) techniques during the early stages of drug discovery thereby adopting more advanced and accurate approaches to standardize phenotyping across studies and have the ability to quickly share and discover data in seconds.

Taking a step toward this goal, the Fallon Lab at Brown University has submitted grant applications to test muscle specific kinase (MuSK) antisense oligonucleotides (ASOs) in 5xFAD mice with the presence and absence of chimeric Aducanumab (chi-Adu), a monoclonal antibody, to show possible removal of plaques via the binding of oligomeric Aβ which induced ARIA in clinical trials (Sevigny et al., 2016).

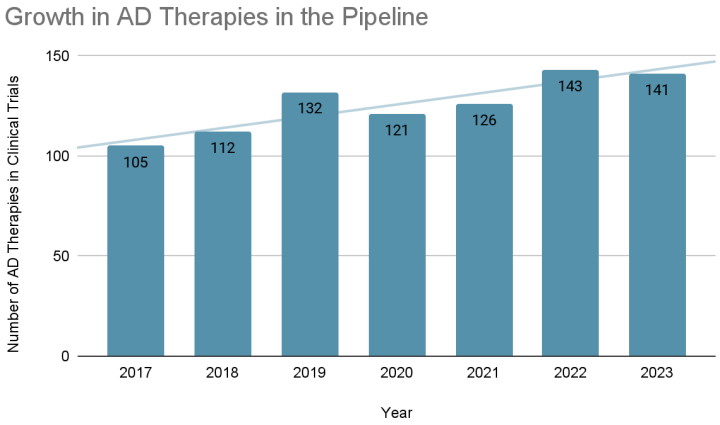


Figure 4.1: Strength in Numbers? There are 36 more therapies being tested in 2023 versus 2017 with a steady increase looking to continue that just might increase the options AD patients have in the future (Cummings et al., 2023)

4.6 | Future Considerations

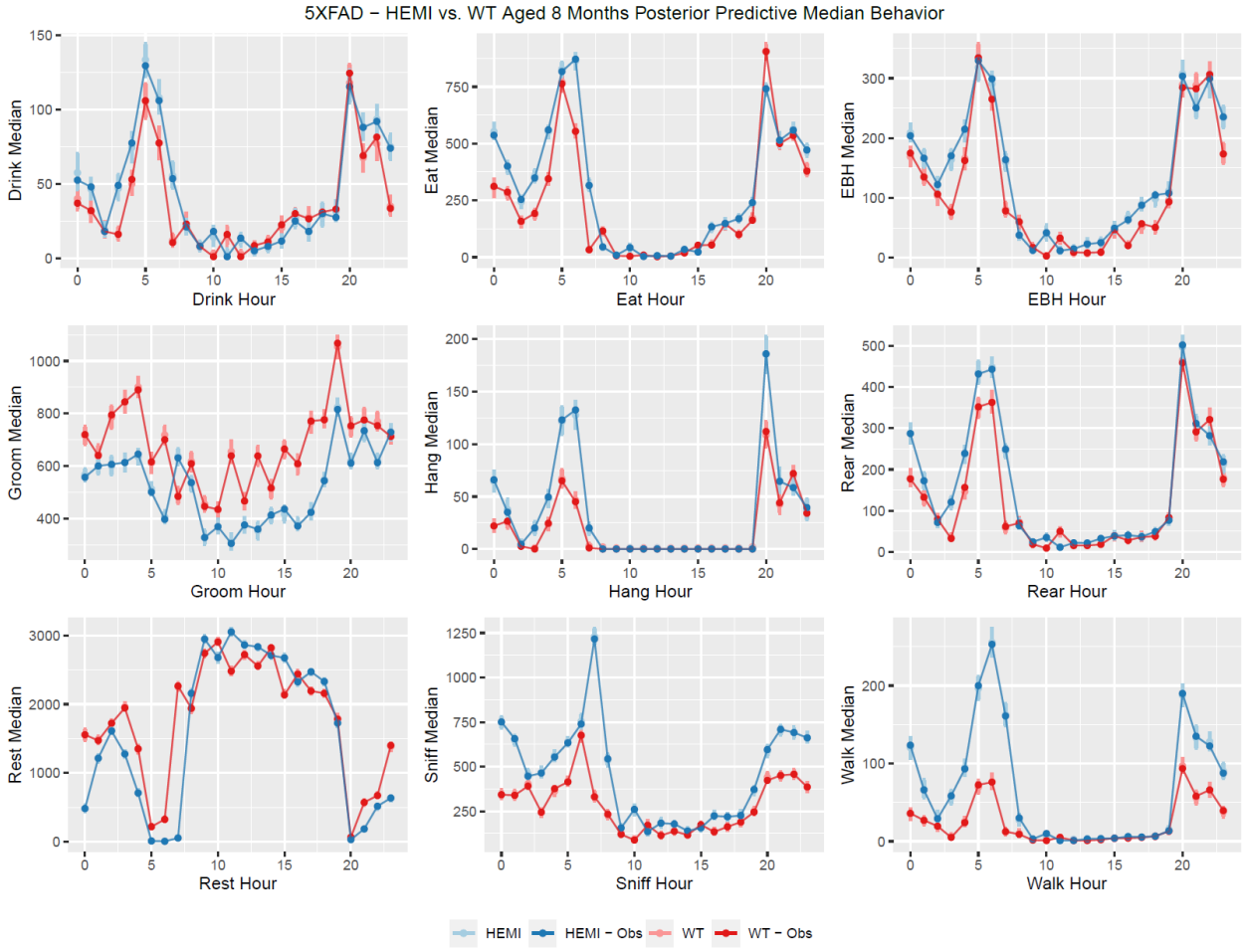
While ACBM provided clear and distinct phenotypes for 5xFAD mice at 8 months, we aim to record 2, 4, 6, and 10 months of age to provide a more comprehensive lifespan of the 5xFAD mouse. Collecting these data will allow us to also make more informed decisions related to other models assessed with ACBM, such as the $Sod1^{G85R}$ mice of ALS.

Additionally, it would be useful to record and analyze other AD mouse models, specifically P301S and P301L, which overexpress human mutant tau (Wenger et al., 2023). While this would

inherently expand our knowledge base, our secure database, it would allow for more experimentation within the early drug development pipeline testing around DTMs.

Lastly, while our original goal was to record 5xFAD mice at 2, 4, 6 and 8 months of age under standard 12 hour light/dark cycles, we experienced multiple infrastructure setbacks that left the finished recordings of 2 and 4 months unusable. First, our team experienced a facilities issue where the lights activated in the room were accidentally switched off leading to full 24 hours of light in both the 2 and 4 month recording. At 6 months, our team received a notification that due to electrical issues the disks saving the data while recording shut down during the active session, leading to a manual restarting of the system and a break in recording time. Our team is in the process of re-recording all of these ages and will utilize the data to draw further conclusions on past studies.

Supplemental Data
Supplementary Figure 1 5xFAD Hemizygous vs. WT ACBM Posterior Predictive Plots



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