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Impact of Long-Term Dietary High Fat and Eicosapentaenoic Acid on Behavior and Hypothalamic-Pituitary-Adrenal Axis Activity in Amyloidogenic APPswe/PSEN1dE9 Mice

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Keywords
Eicosapentaenoic acid · Diet-induced obesity · Alzheimer's disease · Corticosterone · Behavior

Abstract
Introduction: Alzheimer's disease (AD) alters neurocognitive and emotional function and causes dysregulation of multiple homeostatic processes. The leading AD framework pins amyloid beta plaques and tau tangles as primary drivers of dysfunction. However, many additional variables, including diet, stress, sex, age, and pain tolerance, interact in ways that are not fully understood to impact the onset and progression of AD pathophysiology. We asked: (1) does high-fat diet, compared to low-fat diet, exacerbate AD pathophysiology and behavioral decline? And, (2) can supplementation with eicosapentaenoic (EPA)-enriched fish oil prevent high-fat-diet-induced changes? ***Methods:*** Male and female APPswePSdE9 mice, and their non-transgenic littermates, were randomly assigned to a diet condition (low-fat, high-fat, high-fat with EPA) and followed from 2 to 10 months of age. We assessed baseline corticosterone concentration during aging, pain tolerance, cognitive function, stress coping, and corticosterone response to a stressor. ***Results:*** Transgenic mice were consistently more active than non-transgenic mice but did not perform worse on either cognitive task, even though we recently reported that these same transgenic mice exhibited metabolic changes and had increased amyloid beta. Mice fed high-fat diet had higher baseline and post-stressor corticosterone, but diet did not impact cognition or pain tolerance. Sex had the biggest influence, as female mice were consistently more active and had higher corticosterone than males. ***Conclusion:*** Overall, diet, genotype, and sex did not have consistent impacts on outcomes. We found little support for predicted interactions and correlations, suggesting diet impacts metabolic function and amyloid beta levels, but these outcomes do not translate to changes in behaviors measured here.

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Introduction

Alzheimer's disease (AD) has been traditionally defined by impaired cognitive function as well as the presence of amyloid beta plaques, neurofibrillary tau tangles, and loss of neurons in memory-associated brain regions [1–3]. Despite major investment in AD research, the etiology of this disease, and the factors that enhance progression and accelerate physiological and psychological decline, are still not well understood. Recent work highlights that AD is a whole-body and multifactorial disease, impacting many physiological systems and influencing multiple types of behaviors, not just those associated with cognition [4]. For example, AD is associated with alterations in affective state (e.g., anxiety, depression, irritability; [5]), pain sensitivity [6], metabolism [7, 8], and the physiological and psychological response to stressors [9]. Additionally, many health conditions influence AD, e.g., hypertension, hypercholesterolemia, chronic inflammation, obesity, and type II diabetes are all associated with an increased risk of cognitive decline, dementia, and AD pathology [10–13]. Relatedly, AD has recently been labeled type III diabetes, as insulin resistance, neural inflammation, as well as systemic and brain metabolic dysregulation are important factors in AD pathophysiology [14, 15].

The relationship among stressor exposure, hypothalamic-pituitary-adrenal (HPA) axis hormones (e.g., glucocorticoids), and AD has been formalized into the concept of the vicious cycle of stress [9]. The framework posits that stress drives disease and disease causes stress, thus creating a positive feedback cycle [9]. This paradigm fits well with the glucocorticoid cascade hypothesis (GCH) [16–19], which states that elevated glucocorticoids impair negative feedback mechanisms in the brain, resulting in less effective negative feedback regulation and therefore higher glucocorticoids [16]. These elevated glucocorticoids then lead to neuronal loss, further impairing feedback, and negatively impact cognitive behavior [16, 19]. In support of this, many studies have found that both human patients and animal models of AD exhibit elevated baseline glucocorticoids, altered HPA response to stressors, and/or dysregulated negative feedback.

Obesity alone, especially in midlife [20], or in combination with high-fat diet, can alter aspects of physiology, including those involved in AD onset and progression, such as pain, inflammation, blood pressure, glucose handling, lipid balance, and HPA axis activity [21, 22]. Obesity and high-fat diet consumption can decrease cognitive function and increase glucocorticoids and inflammation [23]. In both humans and animals, consumption of a fat-rich diet and/or obesity is associated with increased activity of the HPA axis [24–26]. For example, diet-induced obesity through high-fat feeding (ranging from 2 to 30 weeks) increases circulating baseline glucocorticoids in mice [25]. High-fat diet can also result in elevated post-stressor glucocorticoids [27]. Thus, increases in baseline and post-stressor glucocorticoids following high-fat diet and in obesity are concerning as both factors can negatively impact AD pathophysiology [28] and cognitive function. It seems likely that the HPA axis, diet, and metabolic function play an interacting role in AD onset, progression, and/or severity.

The specific type of fatty acids in the diet, that is saturated or unsaturated, also plays a role [26]. Saturated fatty acids are most problematic and supplementation with unsaturated fatty acids can reverse or ameliorate the negative metabolic impacts of high-fat diet. For example, the majority of human and animal studies have focused on the *n*-3 long-chain polyunsaturated fatty acids (PUFAs), typically docosahexaenoic acid (DHA) or a mix of DHA and eicosapentaenoic acid (EPA), with varied results on improving cognitive function [29, 30]. Further, recent reviews discuss potential benefits of *n*-3 PUFA in improving cognitive functions in military members [31] and healthy young adults [32], however, more in-depth research is needed. The effects of solely EPA supplementation in the resolution of AD pathophysiology and behavior have not completely been evaluated, despite the current use of EPA as an effective triglyceride-lowering therapy [33].

We propose that dietary supplementation with EPA can prevent high-fat-diet- and genotype-induced impacts on HPA activity (i.e., baseline and post-stressor glucocorticoid concentrations), cognitive performance, and behavioral outcomes in an amyloidogenic mouse model of AD. The proposed use of dietary EPA to reduce glucocorticoids and improve cognitive function in an AD mouse model is a novel concept and, if successful, could provide a noninvasive, simple, and safe, dietary approach for reducing or halting adverse effects of AD-associated pathophysiology on organismal function. EPA and fish oil are already used clinically as hypotriglyceridemic agents (e.g., prescription strength combined EPA and DHA or only EPA formulations [34]). Thus, our work could provide additional scientific evidence for the pleiotropic benefits of these PUFAs. Lastly, given that sex differences are important for physiological and behavioral processes [35–38], and for disease outcomes, including in AD [35, 39, 40] and response to high-fat diet [41], we used both male and female mice.

Several mouse models of AD exist, including strains with mutated or altered amyloid precursor protein (APP), presenilin 1 (PS1), microtubule-associated protein tau (Mapt), and apolipoprotein (Apoe) [42–44], however, no single model fully captures all pathophysiological or behavioral aspects of AD. For this study, we used the APPSwePSEN1 line of mice. This line expresses chimeric mouse/human APP and a mutant version of the human PS1 in CNS neurons [45, 46]; both mutations present in this transgenic line are associated with early-onset AD in humans. APPswePS1dE9 mice develop brain beta amyloid deposits starting as early as 3–4 months with pronounced deposition in the hippocampus by 9 months [47–49]. This line does not develop tau tangles. Cognitive deficits are apparent as early as 4 months of age [50] with pronounced decline at older ages, especially after 6–9 months [44, 45]. These mice are one of the most used Alzheimer's models [44, 51], which allows us to place our behavioral and physiological data within the context of other studies. Additionally, given that glucocorticoids, high fat diet, and obesity-associated sequela are all associated with increased inflammation and amyloid beta pathophysiology [9, 52, 53] and are risk factors for AD, this mouse model allows us to ask specific questions about those relationships.

Specifically, we asked: (1) does high-fat diet, compared to low-fat diet, exacerbate AD pathophysiology and behavioral decline? And, (2) can supplementation with EPA-enriched fish oil prevent high-fat-diet-induced changes in AD pathophysiology and behavior? Male and female APPswePS1dE9 transgenic mice, and their non-transgenic littermates, were fed different diets (low-fat, high-fat, high-fat with EPA) from 2 to 10 months of age. During that time, all mice underwent several procedures to assess behavior and HPA axis function. A subset of the physiological data from these same mice, including amyloid beta in serum and brain, has been reported recently [54]. Here, we report on behavioral and HPA axis outcomes. During the 8-month study, a total of four different behavioral assays were conducted: novel object recognition (NOR) task (recognition memory; age 6 months), hot plate test (pain tolerance; age 8 months), Morris Water Maze (MWM; spatial memory; age 9 months), and forced swimming task (stressor test and behavioral despair assessment; age 10 months). Blood samples (6 per mouse over the experiment; 5 baselines and one post-stressor) were collected for plasma corticosterone analysis. We made several specific predictions about the way in which diet, genotype, sex, and age would impact outcomes.

Methods

Animals

Mice for this experiment were bred and housed at Texas Tech University and were descendants of breeding pairs purchased from Jackson laboratory in 2016. Original breeding pairs were from the B6.Cg-Tg(APPswe, PSEN1dE9)85Dbo/Mmjax line, and consisted of transgenic males (stock 005864; Genotype: hemizygous for Tg(APPswe, PSEN1dE9)85Dbo) and noncarrier females (stock 005864, Genotype: Noncarrier, JAXEAST:AX10). All subsequent breeding was done in-house using a non-transgenic female and a transgenic male. Importantly, to control for maternal effects and non-target impacts of genotype, we used non-transgenic littermates, versus separately purchased wild-type C57BL/6J mice, as our controls.

Mice were weaned at approximately 26 days of age and housed in same-sex groups of four until genotyping. Genotyping was conducted via PCR using tail snip tissue; methods are described in [54]. Sex and genotype information was used to assign mice to diet conditions (originally, 10–11 mice per group). Upon assignment, mice were individually housed in 14" × 8" × 5.5" polysulfone cages (Blue Line 1285L) with corn cob bedding, a plastic nesting hut, wooden chew cubes, and free access to tap water. To track individual food consumption, mice remained singly housed throughout the remainder of the experiment. Cages were maintained in ventilated racks (Blue Line); room temperature was kept between 22 and 23°C. Lights were on a 12:12 L:D cycle; lights on at 08:00 h. Room traffic and noise were kept to a minimum and were consistent across the experiment. A total of 133 mice entered the protocol and seven transgenic mice died naturally over the course of the 10-month study (one LF Tg male; 1 HF Tg female; 1 HF Tg male; 4 HF-EPA Tg females); remaining sample sizes per experimental group were 8–11 mice. To accommodate intensive behavioral testing, mice were tested in cohorts. A total of 11 cohorts with 8–15 mice each completed the study. Experimental conditions were balanced within cohorts.

Diets were prepared by Research Diets, Inc. (V10001, AIN-76A Vitamin Mix, New Brunswick, NJ, USA), and each diet was dyed a different color. Diet conditions were low fat (LF; 10% kcal fat, 20% kcal protein, and 70% kcal carbohydrates; 3.85 kcal/g), high fat (HF; 45%, 20%, and 35% of energy from fat, protein, and carbohydrates, respectively; 4.7 kcal/g), and a high fat supplemented with EPA as 36 g/kg of EPA ethyl ester enriched fish oil (45% fat, 20% protein, and 35% carbohydrates; 4.7 kcal/g). Alaskomega® EPA-enriched fish oil contained 700 mg EPA/g fish oil and was kindly provided to us by Wiley Companies (Coshocton, OH, USA). Detailed diet information about diets can be found in [54]. Mice were provided preweighed amounts of food and food hoppers were checked regularly; if the hopper was empty, new food was weighed and the hopper was replenished. The amount (in grams) of food consumed by each mouse was recorded weekly; results of food consumption and body mass are presented in [54]. The data reported here is part of a larger study using these same animals [54], but dependent variables presented here are unique. TTU has AAALAC accreditation, and all procedures were approved by the TTU IACUC (IACUC protocol number: T19040) and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

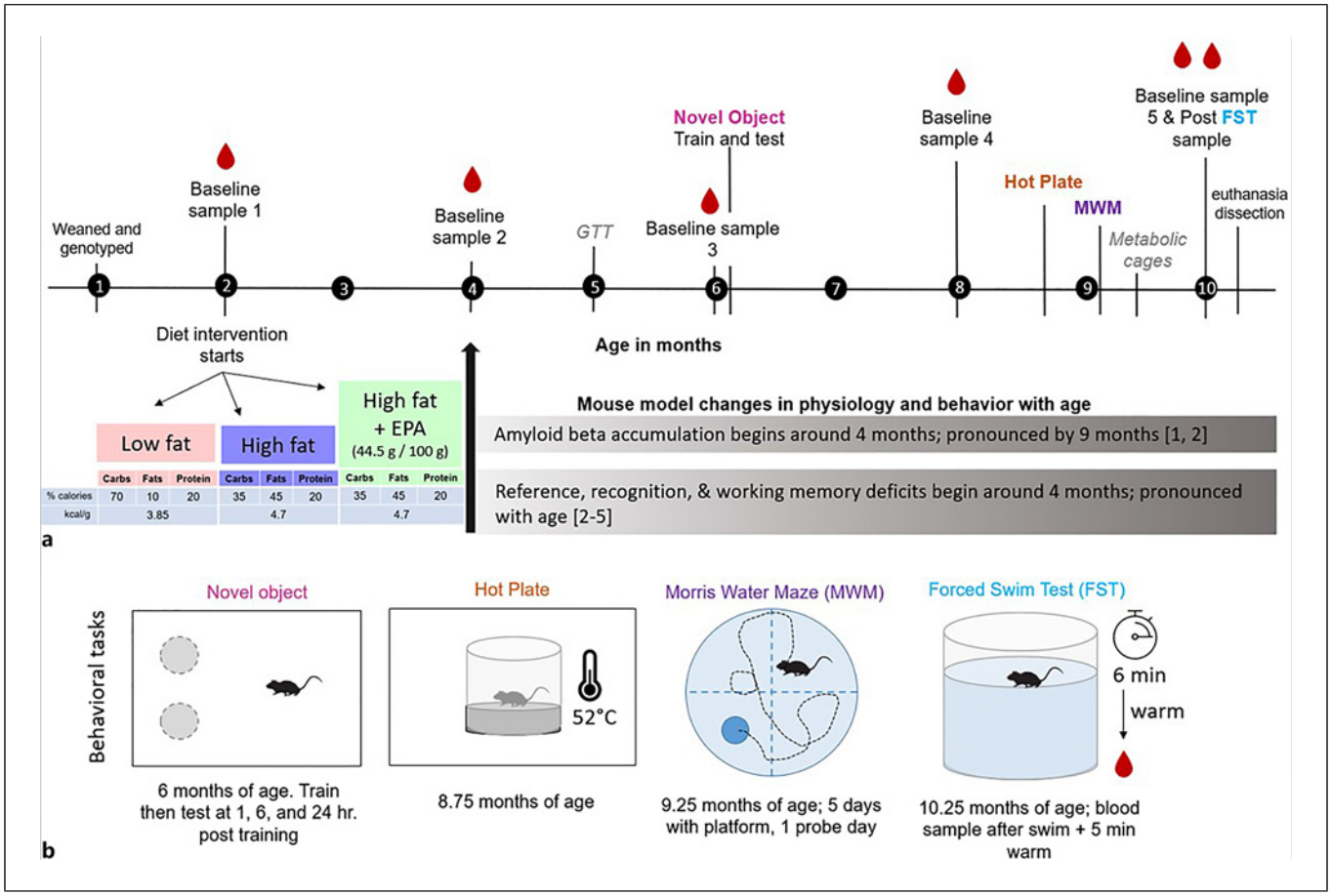

Fig. 1. a Experimental timeline showing blood sample collection and behavioral tasks for all mice in the study. Data for items listed in grey (glucose tolerance testing; GTT and metabolic cages) are presented in our other paper [54]; body composition of mice was also assessed throughout the study, those data also in [54]. This mouse model begins to develop amyloid plaques and behavioral deficits around 4 months of age, with severity increasing with age. **b** The four behavioral tasks completed in this study; each mouse completed each task (References in **a**: 1 [47], 2 [48], 3 [44], 4 [55], 5 [49]).

Experimental Timeline

Mice in each of the twelve experimental groups entered the study protocol at approximately 65 days of age (range: 51–83), at which time they were assigned to a diet condition and continued that diet until euthanasia at approximately 314 days of age (range: 297–333); mean duration on diet was 250 days (range: 227–262). Throughout the 8-month study period, mice were exposed to four behavioral tasks and underwent 6 blood sampling sessions (see Fig. 1). Mice also underwent glucose tolerance testing, metabolic cage monitoring, and routine body composition analysis, but those data are presented elsewhere [54]. For all behaviors, testing sessions were video recorded and later scored using EthoVision XT software (Noldus, Inc.). Specific behavioral procedures are described below.

Behavioral Tasks

Novel Object Recognition (NOR) Task

The NOR task is commonly used to assess recognition memory in rodents and relies on rodents' ability to recall familiar objects after either short- (minutes to hours) or long-term (hours to days) separation [56]. This test is particularly relevant for studying cognitive deficits in animal models of human disorders [57], and deficits in this task have been observed in APPswePS1dE9 mice by 6 months of age [49].

The arena was an opaque, white, square open plastic box measuring 34.3 × 34.3 centimeters. Inside the arena, areas were marked with marker to designate novel object placement locations. We had two arenas for males and two arenas for females; arenas and objects were wiped with 70% ethanol between trials to eliminate conspecific odors. All behavioral trials were 10 min in duration and were video recorded. Mice were exposed to the arena four times: at training and again 1-h, 6-h, and 24-h post training. During the training phase, mice were given two identical objects to investigate (steel whisky ice cubes; see online suppl. Fig. 1; for all online suppl. material, see https://doi.org/10.1159/000536586). During the testing phases (1, 6, and 24 h later), the mice were given one training object and one novel object (novel object was different at each testing time point; 1 h, cone-shaped steel ice cube; 6 h, glass aquarium shell; 24 h black whiskey ice

cube; online suppl. Fig. 1). The location (R or L) of the novel object was balanced to control for placement. All data collection occurred during lights on using the following schedule: 9:00 a.m. training, 10:00 a.m. test (1 h), 4:00 p.m. testing (6 h), and 10:00 a.m. test (24 h).

Videos were scored with Ethovision to determine the duration of time spent investigating each object (nose on or within 2 cm of the object) throughout the duration of the trial and recognition index was calculated as the time spent investigating the novel object relative to the total object investigation [RI = $T_N/(T_N + T_F)$ *100]; ranging from 0 to 100. A score of 50 would indicate no preference for either object, whereas values over 50 suggest preference for the novel object (e.g., recognition of the familiar object). We also determined distance travelled in the arena during the test; distance data were log-10 transformed to improve normality. All data were analyzed by 2 (sex) × 2 (genotype) × 3 (diet) ANOVA for each testing time period.

Hot Plate Task

This behavioral task is commonly used to assess nociceptive responses in various species of rodents [58–61]. Mice were placed onto a clean hot plate and covered with a plexiglass cylinder (6 cm height × 20 cm diameter) with air holes drilled into it. Hot plate temperature was maintained at 52.0 ± 0.2°C and testing lasted 30 s; testing occurred between 10:00 and 11:00 a.m. Mice were monitored throughout the test and were removed from the hot plate if they exhibited the nociceptive behaviors of shaking, licking, or sustained lift of hind paws (only occurred 1 time). Video recordings were imported into Ethovision and manually scored. Latency to exhibit any of the above behaviors was recorded and used in analysis. Mice that never exhibited nociceptive behaviors were assigned a score of 30 s. All data were analyzed by 2 (sex) × 2 (genotype) × 3 (diet) ANOVA using SPSS.

Morris Water Maze (MWM)

The MWM is a common method for assessing hippocampal-dependent spatial learning and long-term spatial memory in rodent models of AD [62–64], and APPswePS1dE9 mice show deficits in this task starting at 6 months of [44]. Our experimental arena was a blue circular pool (internal diameter 122 cm, water depth 59 cm; BehaviorCloud LLC, Columbus, OH, USA) placed inside a custom-build 5 ft × 5 ft PVC frame; a GoPro camera was mounted directly above the pool. Black curtains were hung from the frame and custom-made, high contrasting spatial visual cues were affixed to the sides of the pool and above the tank on the curtains and remained present for the entirety of the experiment (online suppl. Fig. 2).

To standardize both platform and mouse locations, the tank was visually divided into four equal quadrants (NE, SE, SW, NW). A clear plastic platform (BehaviorCloud LLC, Columbus, OH) was placed in the SW quadrant in the same location for all testing days. Mice swam in a pool of clear (day 1; training) or opaque (days 2–6; testing) water to find a platform. Throughout the trials, the mouse should locate a visible and marked (day 1), submerged (days 2–5), or missing (day 6) platform (location) by using learned visual cues. Water temperature was 25–26°C and one white tempera paint cake (2 ¼ × ¾ inch) was used to dye water for days 2–6. After each mouse completed the daily trials, it was lightly towel dried and placed in a cage with a heating pad to warm for approximately 7 min.

Over the course of six consecutive days, spatial learning and memory were assessed by placing the mouse in pseudorandom predetermined starting locations of the pool using a previously published schedule [65]. Days 1–5 consisted of 4, 60-s trials per mouse per day, and day 6 was a single 60-s probe trial. Day 1 was a training day with clear water and a flagged and visible platform. Days 2–5 were testing days with opaque (white) water and a submerged platform. Behavioral tests were video recorded and scored for latency to find the platform (escape latency; days 1–5, training and testing) or platform location (duration in quadrant; day 6, probe). If the platform was not found upon the completion of the 60-s trial in days 1–5, the mouse was gently guided to the platform; for all trials, once the mouse was on the platform it was given a 20-s acquisition period to assess its environment. For days 1–5, duration of time to find the platform was recorded and for each day, a single average score from the 4 trials was calculated. For days 1–5, if the mouse did not locate the platform during a trial, the mouse was given the max time of 60 s for analysis. Day 6 was a probe trial with opaque water and no platform. For the probe trial, the duration of time spent in the quadrant previously containing the platform was recorded. Average distance traveled, average duration to reach the platform, and mean velocity each day for days 1–5 were analyzed using a repeated-measures ANOVA with time as the within-subjects repeated factor (trial day) and diet type, genotype, and sex as the fixed, between subjects' factors. Probe data (duration of time in SW quadrant, entries into the SW quadrant, distance traveled, and mean velocity) were analyzed via a 3 (diet) × 2 (genotype) × 2 (sex) ANOVA.

Forced Swim Task

A 1-day version of the forced swim task (FST) was used, following general methods described elsewhere [66]. Mice were tested individually and placed into a 22.8 cm diameter plastic cylinder (Buddeez, Inc. Wentzville, MO, USA) full of tap water (average temperature 24.4°C, range 23.9–25.2) for a total duration of 6 min. The volume (6.6 L) and depth of water were kept consistent across trials and water was deep enough so the mouse's tail did not touch the bottom while the mouse was swimming or floating. Immediately following the 6-min swim, mice were placed in a holding cage with a towel and a heat lamp for 5 min to dry and warm. Next, mice were anesthetized with isoflurane for collection of a blood sample (see below).

The swimming session was video recorded for later behavioral scoring using Ethovision (the last 4 min of the test were used to score immobility; see Can et al. [66]). For Ethovision scoring (v14, Noldus, Inc.), we used the setting specific for the FST and used output from the Motility State 30 setting (average sample interval: 30; high mobility about 60% and immobile below 30%; mobile 30–60%). The data (time spent immobile in seconds, out of a possible 240) were analyzed via 2 (sex) × 2 (genotype) × 3 (diet) ANOVA using SPSS. Given that body mass differed systematically across treatment conditions (sex and diet), we also ran our analysis with body mass as a covariate in case this variable influenced floating behavior.

Blood Sample Collection

A total of six blood samples were collected from each mouse (five baseline samples at 2, 4, 6, 8, and 10 months of age; 1 post-stressor sample at 10 months of age), resulting in a total of 767 blood samples for the experiment. Baseline samples were collected from mice taken

Table 1. Specific questions and predictions for the impacts of genotype, diet, sex, and age on behavioral and physiological outcomes over the 8-month diet-intervention study

Questions and predictions	Outcome
1) Do corticosterone concentrations differ between transgenic and non-transgenic mice with age, by sex, and by diet?	
Diet will impact baseline corticosterone (main effect of diet; HF > LF and HF-EPA)	Supported
Genotype will impact baseline and post-stressor corticosterone (Tg > non-Tg overall)	Not supported
Baseline corticosterone will increase with age (main effect of age; GCH)	Not supported
Sex will impact baseline and post-stressor corticosterone (main effect of sex; female > male)	Supported
Baseline corticosterone will increase with age will interact with genotype (Tg > non-Tg, at 8 and 10 months) and diet (HF > LF and HF-EPA starting at 4 months)	Not supported
Swim stressor will increase corticosterone (main effect of time)	Supported
Post-stressor corticosterone will interact with genotype (Tg > non-Tg) and diet (HF > LF and HF-EPA)	*Partial (diet; HF > LF & HF-EPA)*
2) Does performance on the NOR (recognition index*) differ between transgenic and non-transgenic mice, by sex, and by diet?	
Genotype will impact performance (main effect; Tg < non-Tg)	Not supported
Diet will impact performance (main effect of diet; HF < LF and HF-EPA); interact with genotype (HF Tg mice will perform worst)	Not supported
Diet will interact with genotype and sex (three-way; for HF, Tg < non-Tg and M < F; for LF and HF-EPA, Tg < non-Tg, and no sex diff)	Not supported
*Diet, genotype, and sex all influenced distanced traveled in the novel object task, but this was not a major a priori prediction	*See results*
3) Does performance on the hot plate task (latency and response) differ between transgenic and non-transgenic mice, by sex, and by diet?	
Genotype will impact performance (main effect; Tg < non-Tg)	Not supported (*opposite for response*)
Diet will impact performance (main effect; HF < LF and HF-EPA)	Not supported
Diet will interact with genotype (HF Tg will perform worst)	Not supported
Diet will interact with genotype and sex (three-way; for HF, Tg < non-Tg and M < F; for LF and HF-EPA, Tg < non-Tg, and no sex diff)	Not supported
4) Does performance on the MWM task (escape latency) differ between transgenic and non-transgenic mice, by sex, and by diet?	
Performance will increase over days of the task (main effect of day)	Supported
Genotype will impact performance (main effect; Tg < non-Tg)	Not supported
Diet will impact performance (main effect of diet; HF < LF and HF-EPA)	Not supported
Diet will interact with genotype (HF Tg will perform worst)	Not supported
Diet will interact with genotype, sex, and time (four-way interaction; for HF, Tg < non-Tg and M < F and will not improve as much over time; for LF and HF-EPA, Tg < non-Tg, and no sex difference and will improve over time)	*Partial (four-way was significant but post-hocs not consistent, see text)*
5) Does time immobile in FST differ between transgenic and non-transgenic mice, by sex, and by diet?	
Genotype will impact performance (main effect; Tg > non-Tg)	Not supported (*opposite; Tg < non-Tg*)
Diet will impact performance (main effect of diet; HF > LF and HF-EPA; LF and HF-EPA do not differ)	Not supported (*LF > HF-EPA*)
Diet will interact with genotype (HF Tg will spend the most time immobile)	*Partial (w/in Tg, HF > HF-EPA; LF > HF-EPA)*
Diet will interact with genotype and sex (three-way; for HF, Tg < non-Tg, and M < F; for LF and HF-EPA, Tg < non-Tg, and no sex diff)	Not supported

Table 1 (continued)

Questions and predictions	Outcome
6) Are corticosterone, recognition memory, spatial memory, and body mass correlated?	
Higher body mass, higher (baseline and/or post-stress) corticosterone	Not supported (opposite at several times)
Higher body mass, lower recognition memory	Not supported (but ↑ BM, ↓ distance in NOR)
Higher body mass, lower spatial memory	*Partial (only on day 2 of MWM)*
Higher body mass, shorter latency in hot plate test	Not supported
Higher corticosterone, lower recognition memory	Not supported (but ↑ cort, ↑ distance)
Higher corticosterone, lower spatial memory	Not supported
Higher baseline and post-stressor corticosterone, more time immobile	Not supported

from their home cage and were otherwise undisturbed; post-stressor samples were collected immediately following the 11-min forced swim procedure (above). Mice were anesthetized via isoflurane inhalation and a ~70 μL blood sample was collected from the retro-orbital sinus. Blood samples were almost entirely collected within 3 min of disturbing the cage (baseline) or after swim protocol (post-stressor), with an average of 121.3 s (range: 64–365). Overall, samples were collected between 10:00 a.m. and 5:30 p.m. (70% of samples between 10 and noon; 28% between noon and 2:00 p.m.; and the remaining 2% between 4 and 5:30 p.m.; see online suppl. Fig. 3, 4). Lights were on a 12:12 cycle with lights on at 08:00 h. The post-stressor sample was collected 1–6 days after the 5th baseline sample with an average of 2.3 days. Time of day of post-stressor sample collection was roughly matched to time of day for the 5th baseline; average within 52 min of sample 5 (minimum 0 min, max 2.74 h; all samples collected between 10:30 a.m and 1:10 p.m.). Average age at 6th (final; post-stress) blood sample was 310.4 days (range: 294–326).

Corticosterone Assay

Plasma samples were assayed using Arbor Assay Detect-X Corticosterone EIA kits following the kit instructions and as we have previously done with samples from APPswePS1dE9 mice [67]. A total of 24 assay plates were run, and plates were balanced to include a mix of genotypes, sexes, and diet treatments so as not to introduced systematic error. All samples from a single mouse were diluted 1:100, run in duplicate and were run on the same plate. An internal pool of plasma was run as a control in each plate, intra-assay CV was 3.74% and interassay CV was 11.9%, the standard curve ranged from 39 to 10,000 pg/mL. Plasma corticosterone levels were log-10 transformed for analysis to reduce skew, but untransformed values are presented for ease of interpretation. Plasma corticosterone concentration is expressed in ng/mL. Baseline and post-stressor corticosterone data were analyzed via separate RM ANOVA with time as a repeated measure and 2 (sex), 2 (genotype), 3 (diet) as fixed effects using SPSS.

Analysis

All data were analyzed in SPSS using repeated-measured ANOVA, three-way ANOVA, or Pearson's correlation. Main effects and interactions are reported; effects sizes are reported as partial eta squared. Post hoc tests were performed if main effect or interaction term was significant. Results were considered significant if $p < 0.05$. Several a priori predictions were made (Table 1) and therefore post hoc alpha correction was not used. We also present effect sizes to aid in interpretation of relationship strength. See above sections for specifics about which tests were used for each variable.

Results

Behavioral Tasks

Novel Object Recognition

A total of 127 mice underwent NOR testing (HF = 42; HF-EPA = 42 LF = 43) and mice were between 180 and 214 days of age at NOR training day (average = 195). Distance traveled data were log10 transformed to improve equality of variance across groups.

NOR – Recognition Index and Distance Traveled

The same outcomes were noted for each time point and at no time did any main effect (genotype, diet, sex) impact recognition index (all $p_s > 0.05$; online suppl. Table 1; Fig. 2a–c); nor were any interaction terms significant (all $p_s > 0.17$). Similarly, the distance traveled at 1-, 6-, and 24-h time points showed the same outcomes; at all time points, transgenic mice covered more distance that did non-transgenic mice, and females traveled farther than males (online suppl. Table 1; Fig. 2d–f); none of the interaction terms were significant (all $p_s > 0.05$).

Hot Plate Test

A total of 127 mice underwent hot plate testing (HF = 42; HF-EPA = 42; LF = 43) and mice were tested between 249 and 285 days of age (average = 265); data from one HF mouse were not useable due to logistical issues. Latency data were log10 transformed to increase equality of variance. Not all mice displayed nociceptive behaviors, and thus we performed a χ^2 analysis for diet ($\chi^2 = 2.31$, $p = 0.315$) and for genotype ($\chi^2 = 5.51$,

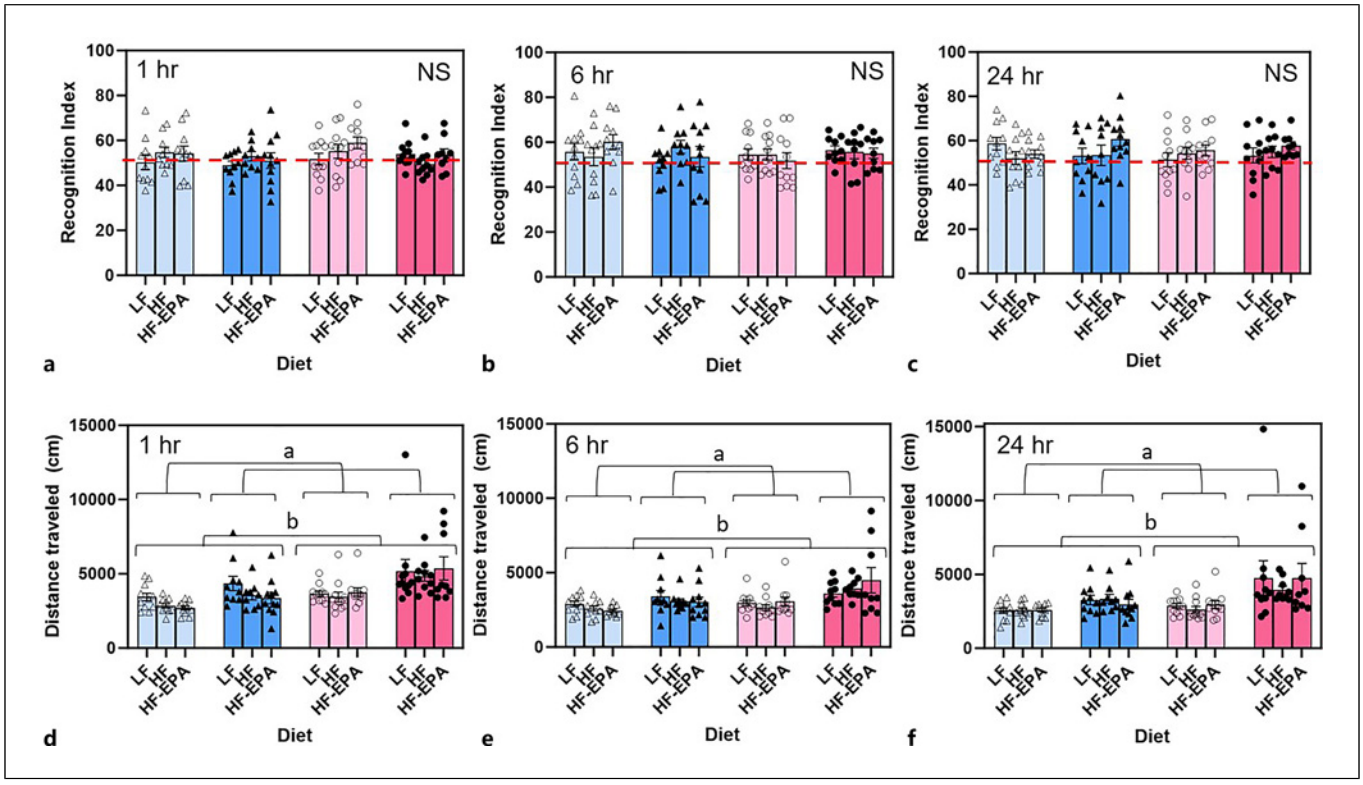

Fig. 2. Mean ± SEM recognition index (**a–c**) and distance traveled (**d–f**) from male (blue, triangles) and female (pink, circles) non-transgenic (open symbols) and transgenic (closed symbols) mice at 1 (**a**, **d**), 6 (**b**, **e**), and 24 (**c**, **f**) h post training in the NOR task. No groups differed in recognition memory performance (**a–c**), however, in all time points tested, transgenic mice (vs. non-transgenics) and female mice (vs. males) covered greater distance during the task (main effects of genotype (**a**) and sex (**b**) at all three time points, letters denote significant differences; **d**–**f**).

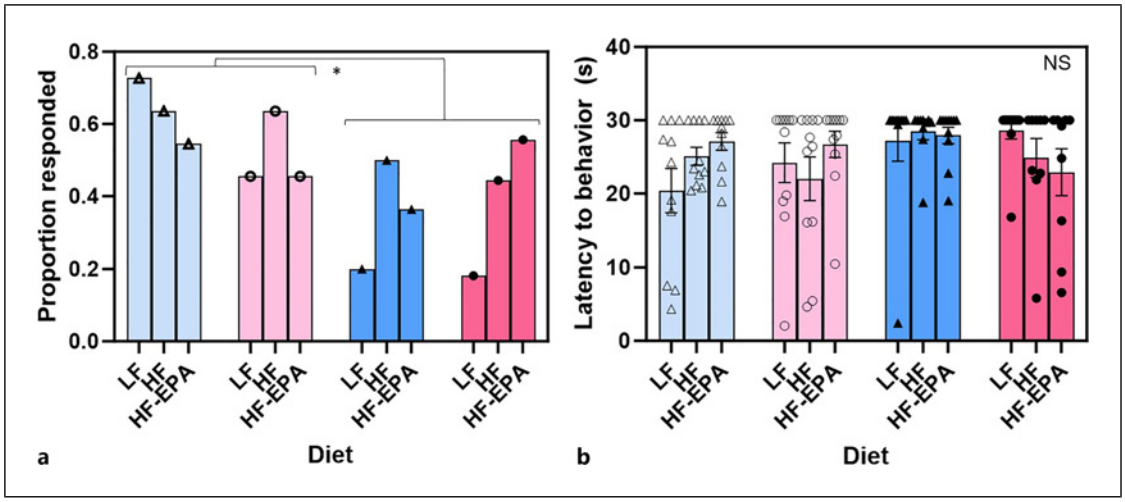

Fig. 3. Data from the hot plate tests. Overall proportion of mice in each group that showed at least one of the nociceptive behaviors during the trial (**a**), a lower proportion of transgenic mice responded (χ^2 on genotype $p = 0.0189$). Mean ± SEM latency to nociceptive behavior (shaking, licking, or sustained lift of hind paws) during a 30 s test on a 52°C hot plate (**b**). If a mouse did not show pain behavior, it was given the max duration of 30 s for analysis. Males are triangles (blue), females are circles (pink), transgenics are filled symbols, non-transgenics are open symbols.

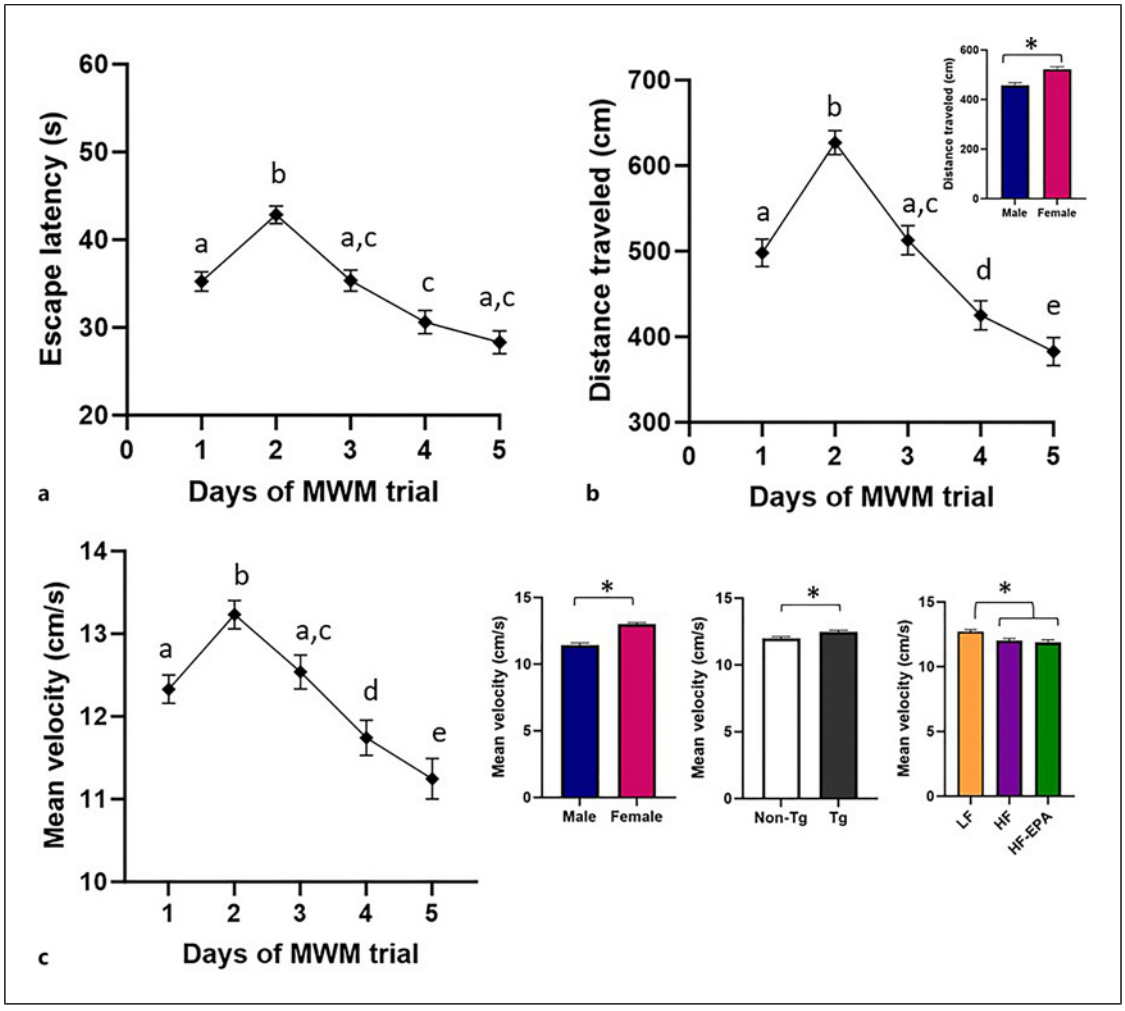

Fig. 4. Mean ± SEM of behavioral data from the MWM trials in male and female mice (day 1 = training day; days 2–5 = test days). Escape latency of all animals combined (**a**), distance traveled of all animals combined (**b**), and mean velocity of all animals combined (**c**) are shown. All three variables decreased over time (time points that share a letter do not differ), but there were no interactions. Main effects of sex (**b**, **c**), genotype (**c**), and diet (**c**), are displayed as insets. For graphs of all data, see online supplementary File 1.

p = 0.0189) to determine if groups differed in proportion of mice responding. Compared to non-transgenic mice, fewer transgenics responded with nociceptive behaviors (Fig. 3a). For our analysis on latency, if a mouse did not respond, they were assigned a value of 30 s. There were no differences across experimental groups in latency to nociceptive behavior in the hot plate test (Fig. 3b); genotype did not impact latency to nociceptive behavior (F(1, 114) = 1.91, p = 0.169, partial eta squared = 0.016), nor did diet (F(2, 114) = 0.71, p = 0.496, partial eta squared = 0.012), or sex (F(1, 114) = 0.76, p = 0.386, partial eta squared = 0.007). None of the interaction terms were significant (all p_s > 0.1).

Morris Water Maze

A total of 127 mice underwent MWM testing (HF = 42; HF-EPA = 42; LF = 43) and at the first training day mice were between 256 and 312 days of age (average = 280). For full MWM data summary, see online suppl. Tables 2–8; Figure 5, and the text provided in online supplementary File 1.

MWM Days 1–5

Escape Latency

Overall, mice improved over time and found the platform faster with more experience (main effect of time; F(4, 460) = 28.88, p < 0.001, partial eta squared = 0.201). Post hoc analyses revealed that day 1 (training day)

escape latency duration was longer than day 4 ($p = 0.002$) and day 5 ($p < 0.001$); day 2 (first testing day) escape latency was longer that every other day (all $p_s < 0.001$), and day 3 (testing day 2) escape latency was longer than days 4 ($p = 0.002$) and 5 ($p < 0.001$); days 4 and 5 escape latency did not differ ($p = 0.082$). Only one other outcome was significant, a 4-way interaction among time, diet, genotype, and sex ($F(8, 460) = 3.46$, $p < 0.001$, partial eta squared = 0.057); however, the post hoc analysis did not reveal any consistent patterns (see online suppl. File 1; Tables 2–4). Data for main effect of time on escape latency are displayed in Figure 4a. No other main effects or interactions were significant ($p_s > 0.05$).

Distance Traveled

Testing day impacted the distance traveled in the MWM pool (main effect of time; $F(4, 460) = 43.31$, $p < 0.001$, partial eta squared = 0.274; Fig. 4b). Post hoc analyses revealed distance traveled generally decreased over days with the only time points that did not differ being day 1 versus day 3 ($p = 0.473$), all other comparisons differed significantly (all $p_s < 0.03$). Females covered more distance than males (main effect of sex; $F(1, 115) = 11.55$, $p < 0.001$, partial eta squared = 0.091; Fig. 4b inset). And again, the only other outcome that was significant was a 4-way interaction among time, diet, genotype, and sex ($F(8, 460) = 3.41$, $p < 0.001$, partial eta squared = 0.056); however, as above, the post hoc analysis did not reveal any consistent patterns (see online suppl. File 1; Table 2, 5, 6). No other main effects or interactions were significant ($p_s > 0.11$).

Mean Velocity

Testing day impacted mean velocity (main effect of time; $F(4, 460) = 23.88$, $p < 0.001$, partial eta squared = 0.172; Fig. 4c). Post hoc analyses revealed mean velocity generally decreased over days with the only time points that did not differ being day 1 versus day 3 ($p = 0.314$), all other comparisons differed significantly (all $p_s < 0.03$). Females had higher mean velocity than males (main effect of sex; $F(1, 115) = 36.40$, $p < 0.001$, partial eta squared = 0.240; Fig. 4c inset). Transgenic mice had a higher average mean velocity than did non-transgenic mice (main effect of genotype; $F(1, 115) = 4.17$, $p = 0.043$, partial eta squared = 0.035; Fig. 4c inset). Diet impacted mean velocity (main effect of diet; $F(2, 115) = 3.64$, $p = 0.029$, partial eta squared = 0.059; Fig. 4c inset). LF-fed mice had higher mean velocity than did HF-fed ($p = 0.026$) and HF-EPA-fed ($p = 0.009$) mice; HF and HF-EPA did not differ ($p = 0.692$). Consistent with the other two MWM measures, a 4-way interaction of time*genotype*sex*diet was significant ($F(8, 460) = 2.14$, $p = 0.031$, partial eta squared = 0.036); again, as above, the post hoc analysis did not reveal any consistent patterns (see online suppl. File 1). No other main effects or interactions were significant ($p_s > 0.11$).

Day 6–MWM Probe Session

None of the experimental variables impacted duration in platform quadrant (Fig. 5a; all $p_s > 0.18$) or the number of times the platform quadrant was entered (Fig. 5b; all $p_s > 0.32$). Females covered more distance than males ($F(1, 115) = 14.79$, $p < 0.001$, partial eta squared = 0.114; Fig. 5c; no other variables impacted outcomes, all $p_s > 0.19$) and had a greater mean velocity ($F(1, 115) = 14.84$, $p < 0.001$, partial eta squared = 0.114; Fig. 5d; no other variables impacted outcomes, all $p_s > 0.19$).

Forced Swim Task

A total of 126 mice underwent forced swim testing (HF = 42; HF-EPA = 41; LF = 43) and mice were tested between 294 and 326 days of age (average = 310). Given that diet and sex can impact body mass and fat mass (Yavari et al. [54]), which can both impact floating, we ran our analysis with and without body mass (results without body mass included can be found in our online suppl. File 1). The effect of body mass was significant ($F(1, 113) = 13.99$, $p < 0.001$, partial eta squared = 0.110; body mass covariate value = 33.87 g) and thus, we left it in the model. Results largely mirrored those when body mass was not included with two notable differences: with body mass included, sex was no longer significant ($F(1, 113) = 0.089$, $p = 0.766$), but diet was now significant ($F(2, 113) = 3.574$, $p = 0.031$, partial eta squared = 0.059; Fig. 6a). HF-EPA-fed mice spent less time immobile than did LF-fed mice ($p = 0.009$); no other diet groups differed. Genotype had a significant effect ($F(1, 13.264)$, $p < 0.001$, partial eta squared = 0.105; Fig. 6a) with transgenic mice spending less time immobile than non-transgenic mice. The diet*genotype interaction remained significant ($F(2, 113) = 4.452$, $p = 0.014$, partial eta squared = 0.073; Fig. 6b). Among transgenic mice, those that were fed HF-EPA diet spent less time immobile than mice on the HF ($p = 0.006$) or LF ($p < 0.001$) diet; HF and LF did not differ ($p = 0.323$). Within the HF-EPA fed mice, non-transgenic mice spent more time immobile that did transgenic mice ($p < 0.001$); no other with-in diet genotype interactions were significant ($p_s > 0.25$). Likewise, the sex*diet interaction also remained significant ($F(2, 113) = 3.458$, $p = 0.035$, partial eta squared = 0.058). Within females, those fed a LF diet spent more time immobile than those fed a HF-EPA diet ($p < 0.001$), and mice fed a HF diet spent

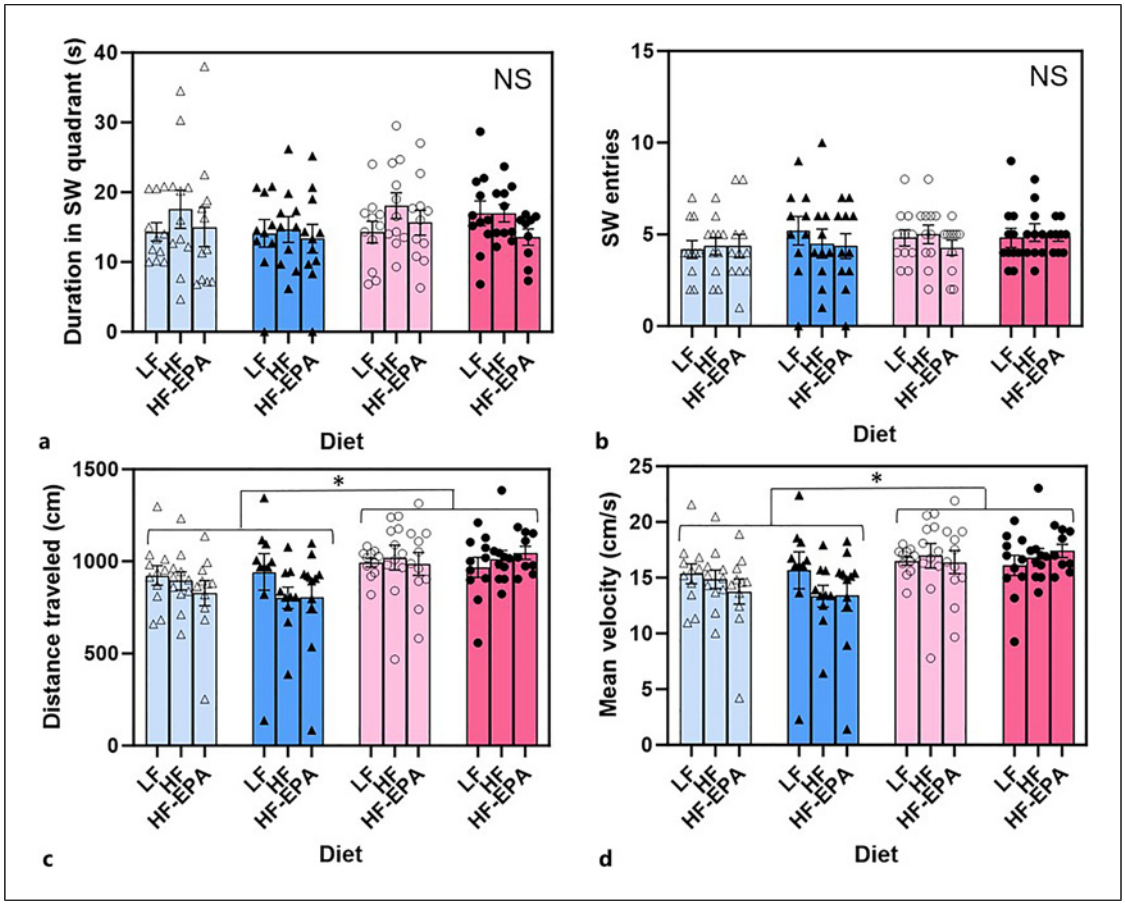

Fig. 5. Mean ± SEM data from the MWM probe trial (day 6) for male (triangles, blue) and female (circles, pink) non-transgenic (open symbols) and transgenic (closed symbols) mice. None of our variables impacted the duration of time in the quadrant that previously contained the platform (**a**) or the number of entries into the quadrant that previously contained the platform (**b**). Females covered more distance than did males (**c**; $*p < 0.05$) and had a higher average mean velocity (**d**; $*p < 0.05$).

more time immobile than those fed HF-EPA diet (p = 0.028; data not shown); no other within sex diet interactions were found (all $p_s > 0.08$).

Corticosterone Data

Out of the 133 mice, a total of 125 had complete corticosterone datasets; for one animal, the third blood sample was lost due to logistical issues and for seven animals, they died prior to finishing the study. Corticosterone data were log10 transformed for analysis, but untransformed data are presented for ease of interpretation.

Baseline Corticosterone during Aging

Baseline corticosterone during aging (measured at 2, 4, 6, 8, and 10 months of age) was impacted by diet (main effect of diet; $F(2, 112) = 5.52$, $p = 0.005$, partial eta squared = 0.090; Fig. 7a, inset) with mice fed a HF diet having overall higher corticosterone than those fed a LF ($p = 0.015$) or HF-EPA diet ($p < 0.001$; LF and HF-EPA did not differ, $p = 0.153$), and sex, as females had higher corticosterone than did males (main effect of sex; $F(1, 112) = 110.93$, $p < 0.00001$, partial eta squared = 0.498; Fig. 7a inset). No other main effects or interactions were significant (all $p_s > 0.05$). Aging had a marginal impact on baseline corticosterone (main effect of time; $F(4, 448) = 2.37$, $p = 0.052$, partial eta squared = 0.021; Fig. 7a), and post hoc analyses show that, overall, baseline corticosterone at 8 months of age was lower than at 2 months ($p = 0.022$) or 4 months ($p = 0.009$) of age; no other ages differed. For graph of log10-transformed corticosterone and the main effect of time, see online supplementary Figure 7.

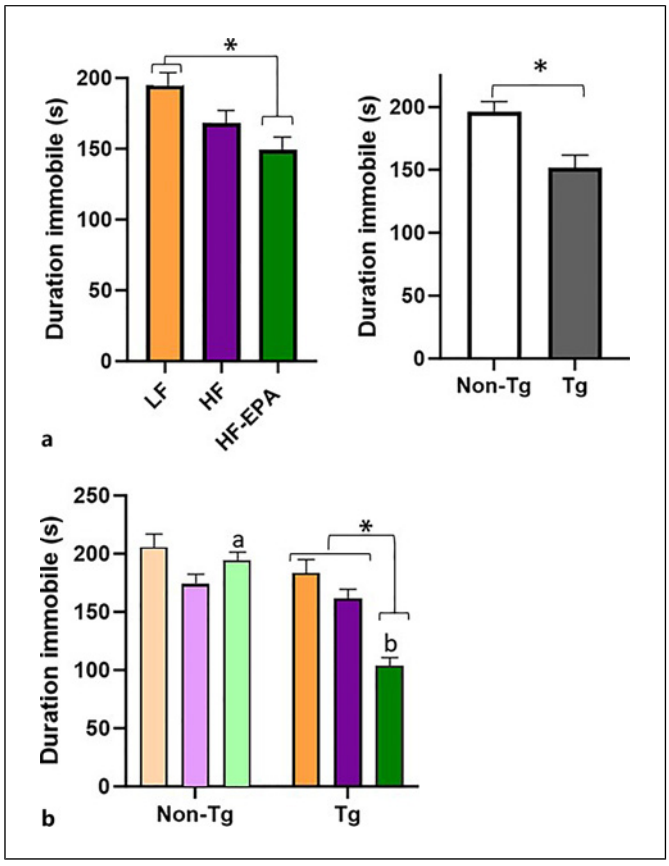

Fig. 6. Mean ± SEM duration of time immobile during minutes 2–6 of the forced swim test; data are presented as marginal means with body mass (33.87 g) used as a covariate. Mice fed the HF-EPA diet spent less time immobile than mice fed a LF diet (**a**; main effect of diet); transgenic mice spent less time immobile than non-transgenic mice (**a**; main effect of genotype); and within transgenic mice, those fed a HF-EPA diet spent less time immobile than did mice fed a HF or LF diet (**b**; genotype × diet interaction, asterisk), and within HF-EPA fed mice, transgenic mice spent less time immobile than did non-transgenic mice (**b**; diet × genotype; **a** vs. **b**; for graph of all data, see online suppl. File 1).

Corticosterone Response to Swimming at 10 Months of Age

Swimming significantly elevated plasma corticosterone (main effect of time; $F(1,114) = 459.98$, $p < 0.001$, partial eta squared = 0.801; Fig. 7b) and, overall, females had higher corticosterone than did males (main effect of sex; $F(1,114) = 45.93$, $p < 0.001$, partial eta squared = 0.287). Corticosterone response to swimming interacted with both sex and genotype (time*sex*genotype interaction; $F(1, 114) = 4.49$, $p = 0.036$, partial eta squared = 0.038), and baseline corticosterone at 10 months of age was higher in male non-transgenic mice than in male transgenic mice ($p = 0.041$; Fig. 7b), but this was not true for females at either time point or for male genotypes at post-swim sample (all $p_s > 0.19$; Fig. 7b). Females had higher corticosterone than males following swim stress for both non-transgenic ($p < 0.001$) and transgenic mice ($p < 0.001$; Fig. 7b); this was also true for transgenic mice for baseline corticosterone ($p < 0.001$; Fig. 7b), but not for non-transgenic mice ($p = 0.183$; baseline corticosterone did not differ between the sexes within non-transgenic mice; Fig. 7b). Lastly, sex and genotype interacted to impact corticosterone (sex*genotype interaction; $F(1,114) = 5.47$, $p = 0.021$, partial eta squared = 0.046; Fig. 7b inset) within males, non-transgenic mice had higher corticosterone than did transgenic mice ($p = 0.033$), but this did not hold in females ($p = 0.248$). None of the other main effects or interaction terms were significant ($p_s > 0.05$).

In addition to the repeated-measured approach to analyzing change in corticosterone from baseline to after a swim stressor, we also analyzed post-swim corticosterone in a separate 2 × 2 × 3 ANOVA. When looking at just post-swim corticosterone, both sex (main effect; $F(1, 114) = 86.39$, $p < 0.001$, partial eta squared = 0.431; Fig. 7c) and diet (main effect; $F(2, 114) = 9.80$, $p < 0.001$, partial eta squared = 0.147; Fig. 7c) impacted corticosterone concentration. Females had higher corticosterone than did males ($F(1, 114) = 86.39$, $p < 0.001$, partial eta squared = 0.431), and mice fed a HF diet had higher post-swim corticosterone than did mice fed LF ($p < 0.001$) and HF-EPA diets ($p = 0.009$); LF and HF-EPA fed mice did not differ ($p = 0.102$). No other outcomes were significant (all $p_s > 0.28$).

Correlational Analysis

We made several a priori predictions about the relationship (correlation) between variables. In addition to our correlations of interest, we ran additional correlations for future hypothesis generation; note these should be interpreted cautiously. None of these a priori predictions were (robustly) supported and the full correlation table can be found in online supplementary Table 9.

We predicted that higher body mass would be associated with higher baseline corticosterone (at 2 months of age: $r = -0.411$, $p < 0.001$, $N = 126$; at 4 months of age: $r = -0.222$, $p = 0.012$, $N = 126$; at 6 months of age: $r = -0.189$, $p = 0.035$, $N = 125$; at 10 months of age: $r = -0.267$, $p = 0.003$, $N = 126$) and higher post-stress corticosterone ($r = -0.222$, $p = 0.012$, $N = 126$); this was not supported and results were to opposite our prediction (higher body mass was associated with lower baseline corticosterone).

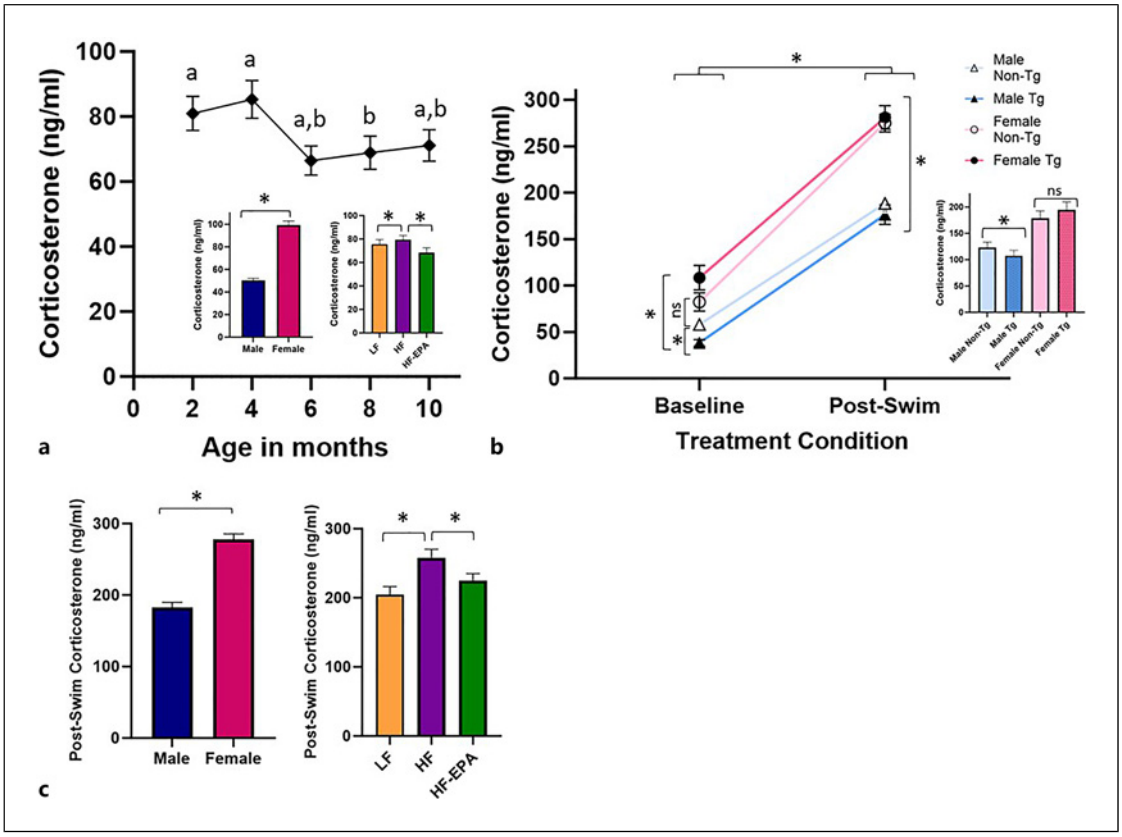

Fig. 7. Mean ± SEM duration baseline plasma corticosterone during aging of all group data combined (**a**) and in response to a swim stressor (**b**; data separated by sex and genotype). Baseline corticosterone was marginally ($p = 0.052$) impacted by age (**a**; time points that share a letter do not differ) and there were main effects of sex and diet (**a**; insets). For post-stressor corticosterone (**b**), swimming significantly increased corticosterone (*baseline vs. post-swim), females had higher baseline and post-swim corticosterone than did males (*circles vs. triangles at baseline and post-stress), non-transgenic males had higher baseline corticosterone than did transgenic males (*open vs. filled triangles at baseline); sex and genotype interacted and overall, transgenic males had lower corticosterone than non-transgenic males (**b**; inset). Upon analysis of just post-swimming corticosterone concentrations (**c**), both sex and diet impacted outcomes (main effects), but no other outcomes were significant. Statistical analyses were run on log10-transformed data but non-transformed data are presented for ease of interpretation.

Due to previous work on obesity and behavioral outcomes, we also predicted higher body mass would be associated with lower recognition memory in the NOR (body mass at 6 months with RA at 1 h: $r = -0.052$, $p = 0.567$, $N = 124$; RI at 6 h: $r = 0.023$, $p = 0.798$, $N = 123$; RI at 24 h: $r = 0.127$, $p = 0.160$, $N = 123$), lower spatial memory in the MWM (latency to platform on day 2 with body mass: $r = 0.205$, $p = 0.021$, $N = 126$; no other associations were significant, all $p_s > 0.139$, see online suppl. Table 9), and shorter latency to show nociceptive behaviors in the hot plate task (body mass and latency score: $r = 0.065$, $p = 0.472$, $N = 125$). Our predictions were not supported as relationships were not consistent.

We predicted higher corticosterone would be associated with lower recognition memory in the NOR (baseline corticosterone at 6 months with RI at 1 h: $r = -0.086$, $p = 0.343$, $N = 124$; with RI at 6 h: $r = 0.013$, $p = 0.890$, $N = 123$; with RI at 24 h: $r = -0.003$, $p = 0.970$, $N = 123$), lower spatial memory in the MWM (baseline corticosterone at 8 and 10 months was not related to any of the latency to platform [days 1–5] or duration in platform quadrant [day 6] data; all $p_s > 0.275$, see online suppl. Table 9), and shorter latency to nociceptive behavior in the hot plate test (baseline corticosterone at 8 months with latency: $r = 0.049$, $p = 0.590$, $N = 125$; corticosterone at 10 months with latency: $r = -0.096$, $p = 0.285$, $N = 125$). We also predicted higher baseline and post-stressor corticosterone would be associated with increased time immobile in the FST (baseline corticosterone at 10 months and immobile: $r = -0.018$, $p =$

0.840, $N = 126$; post-swim corticosterone at 10 months: $r = -0.050$, $p = 0.575$, $N = 126$). Again, our predictions were not supported.

Our exploratory correlational analyses revealed a few notable relationships among variables. Baseline corticosterone at 2 months of age was significantly and positively correlated with baseline corticosterone at 6, 8, and 10 months, and post-swim corticosterone (all $p_s < 0.006$). Distance traveled in the NOR arena at 1 h was significantly and positively correlated with distance traveled at 6 and 24 h ($p_s < 0.001$), and distance traveled in the NOR at each time point was significantly and positively correlated with baseline corticosterone at 6 months of age (all $p_s < 0.032$). However, the recognition index scores at 1, 6, and 24 h were not correlated (all $p_s > 0.422$). Additionally, latency to find the platform in the MWM and NOR RI scores were not correlated (all $p_s > 0.116$). But, starting at day 3, average latency to find the platform in the MWM was positively correlated with latency on subsequent days (all $p_s < 0.001$). Lastly, the immobile duration in the FST was negatively correlated with the distance traveled during day 6 of the MWM ($r = -0.341$, $p < 0.001$), thus, mice that covered more distance in the probe trial also spent less time immobile in the FST. These exploratory correlations mainly suggest individual corticosterone levels and/or behaviors are consistent over time.

Discussion

In this study, we addressed several specific predictions about the impacts of diet, genotype, sex, and age on behavior and HPA axis function in an amyloidogenic mouse model of AD. We hypothesized that transgenic mice would perform worse than their non-transgenic counterparts on various behavioral assessments and that transgenic mice would have higher circulating corticosterone. We further hypothesized that high-fat diet would exacerbate behavioral and corticosterone differences compared to low-fat diet, and that dietary supplementation with EPA would alleviate the impacts of high-fat diet. We predicted sex would impact these relationships. Overall, diet, genotype, and sex did not have major, consistent impacts on the behavioral and physiological outcomes measured here. Surprisingly, transgenic mice did not perform worse on either cognitive task when compared to non-transgenic mice. However, we did find consistent genotype effects on activity levels with transgenic mice being more active than their non-transgenic counterparts. Diet did not impact cognitive or nociceptive behavior, but, consistent with our predictions, mice fed high-fat diet had higher baseline and post-swim corticosterone. Sex had the biggest influence on our data, with female mice being consistently more active and consistently having higher baseline and post-stressor (swim) corticosterone than males. We found little support for multiple predicted interactions, and none of our predicted correlations were robustly supported.

Corticosterone, the HPA Axis, and Glucocorticoid Cascade Hypothesis

Consistent with our predictions, mice fed a high-fat diet had higher circulating baseline corticosterone and higher overall post-swim corticosterone when compared to mice fed a low-fat or high-fat-EPA diet. Given that low-fat- and high-fat-EPA-fed groups did not differ from each other, this suggests dietary supplementation with EPA does ameliorate the impact of high fat diet on corticosterone secretion. The effect of high-fat diet on post-stress corticosterone is consistent with recent data on male (females not tested) C57BL/6J mice fed the same high-fat diet for 8 weeks [68]. We have previously shown that APPswePS1dE9 mice have higher post-stressor (predator odor) but not baseline corticosterone than their non-transgenic littermates, an effect that was evident at 10 months of age, but not earlier in life [67]. We did not find that same outcome here, at 10 months of age, only sex and diet impacted post-stressor (swim) corticosterone. Interestingly, in another study using male and female APPswePS1dE9 mice, we found that at 18 months of age stressor type (odor vs. swimming) mattered for outcomes; transgenic mice had higher corticosterone than non-transgenics when odor was the stressor, but not following swimming [69]. Taken together, these results suggest that age and stressor choice can matter. It is possible that swimming elicits a more robust corticosterone response than does exposure to predator odor (as we have found in California mice, *Peromyscus californicus* [70]), leaving less room for variability (ceiling effect). However, when comparing post-stressor corticosterone levels across our studies on APPswePS1dE9 mice, it does not seem average corticosterone response differs (same assay kits, same age, same time of day, male response ~150 ng/mL and ~300 ng/mL for females). Here, we also found that within males, transgenic mice had *lower* baseline corticosterone at 10 months than did non-transgenics, but this pattern was not found in females, suggesting sex matters. Relatedly, regardless of genotype, we previously found females had higher corticosterone than males at 4 and 10 months of age [67], but not at 2 [67] or 18 months of age [69]. In the

current study, we replicated the sex difference; female mice had higher overall baseline and post-swim corticosterone than did male mice. Also consistent with our previous study [67] corticosterone and behavioral data were not related within individuals, as analyzed via correlational analysis.

Within the GCH framework, we predicted baseline corticosterone would increase with age, and that transgenic mice would have a more pronounced increase. In our previous study [67], we only partially supported the GCH as age did impact corticosterone, but differences were found between 2 and 4 months of age and not 4 and 10 months, suggesting puberty and not aging per se drove the results. Here, we had a more refined sampling time course (e.g., 2, 4, 6, 8, and 10 months of age vs. 2, 4, and 10 months) and could assess nuanced changes with aging. However, corticosterone was only marginally impacted by age and not in the pattern predicted; baseline corticosterone was *lower* at 8 months versus 2 and 4 months of age (no other ages differed). Thus, our data do not appear to robustly support the GCH and even suggest and outcome opposition to predictions; however, we note that the main effect of time was a marginally significant outcome and should be interpreted cautiously. In another study in humans [71], we also did not find robust support for the GCH when assessing baseline cortisol in an aging population, as average cortisol did not increase over time. However, we did find that at the individual level, those with increased cortisol over time performed worse on select cognitive tests over time [71], suggesting large datasets that can target individual variation could be important. The GCH was originally proposed to explain post-stressor (1 h immobilization) corticosterone data in young (3–5 months) versus old (24–28 months) rats [16]; however, increases in glucocorticoids with aging are common in multiple vertebrates (see Table 4 in [72]; and [73]), and the GCH framework has been discussed particularly in the context of AD [9, 18]. Specifically, the relationship of stress and the glucocorticoids on cognitive aging and Alzheimer's-related pathophysiology has been termed the vicious cycle of stress [9]. Despite some inconsistent outcomes between our current work and our previous study on corticosterone, genotype, and aging [67], all of these data are still informative. We explicitly and systematically tested the GCH within the context of diet and genotype and in doing so we were able to assess a wider range of ages to further elucidate relationships among variables. Our data here, combined with previous literature, can add nuance to the understanding of this vicious cycle of stress and address basic questions in stress biology (e.g., why and when do organisms show intra- and inter-individual variation in stress responses and disease susceptibility?), both of which can inform basic and applied research.

Additionally, comparison of our datasets can yield information about experimental variables. Mice from these three studies lived in the same vivarium and were bred from the same stock of transgenic mice at Texas Tech University; light cycle, temperature, humidity, and vivarium traffic were generally consistent between studies, as were blood sampling methods, stressor methods, time of day of sample collection, assay kits, and assayist. However, animal housing and experimental design parameters differed, e.g., single (here and [69]) versus paired [67] housing; weekly (here), daily [69], versus intermittent [67] handling/testing; food delivery (here: portioned out) ad lib hopper provided but pulled approximately 2.5 h before blood sampling [67] and food restricted to keep mice at 85% of adult weight [69]; and diet (here colored pellets from Research Diets, vs. dry, brown pellets from Envigo 2020x [69] and Teklad 2020x [67]). It is not clear if these experimental variables impacted outcomes, but it is well known that various "hidden variables" including light cycle, as well as animal handing, housing, and husbandry can alter neuropsychopharmacology studies, including HPA axis and levels of corticosterone in rodents [74–76]. Interestingly, two recent studies on C57BL/6 mice (the background for our transgenic line) found that for male mice, single housing versus group housing was associated with lower levels of urinary corticosterone, and that establishment of a new housing situation (e.g., group, individual, automated monitoring cages) is associated with changes in corticosterone excretion but alterations are transient, and groups were indistinguishable by 21 days [77]. In another study, housing (single vs. same-sex groups of 3) did not impact baseline corticosterone or behavior in male or female C57BL/6 mice [78]. We are not aware of a study systematically addressing the role of housing numbers and corticosterone in APPswePS1dE9 mice. However, studies on environmental enrichment in mouse models expressing human amyloid beta show that environmental enrichment either elevated corticosterone (male APPswePS1dE9 mice housed in standard housing or housing with extra space and toys [79]) or resulted in no change (TgCRND8 mice of both sexes vs. mice housed individually or in groups in standard cages [80]). In non-transgenic mice, the impact of environmental enrichment on corticosterone and HPA axis function has mixed results [81]. Thus, impacts of housing conditions on corticosterone in C57BL/6 mice and their transgenic derivatives is not clear. The individual housing paradigm

used here was necessary for our experimental design [54] and was applied equally to all mice; therefore, we do not anticipate housing to have differential or confounding impacts on our study animals. However, housing is a variable that should absolutely be considered when comparing data across studies.

Cognitive Function

Interestingly, we did not find evidence that genotype or diet impacted cognitive performance in the NOR or the MWM. Overall, all mice had the capacity to learn using spatial memory (assessed in MWM), but recognition memory performance was low across the board (NOR data). The most consistent outcome from these behavioral tasks was for movement or activity. In both the NOR and MWM, transgenic mice spent more time active (i.e., distance traveled or mean velocity) than did non-transgenic mice. Additionally, females spent more time active than did males.

The lack of genotype effect in the NOR mirror our recent findings using this same line of transgenic mice; we previously found no impact of genotype on NOR when male and female mice were tested at 6 or 8 months of age under baseline and post-stressor conditions [67]. Our results are also consistent with those of other groups using these mice. For example, Bonardi and colleagues, found no impact of genotype (APPswePS1dE9 vs. non-tg littermates) in the NOR when 4-month-old female mice (males not used) were tested; data were presented as percentage of time exploring the novel object (both genotypes ~60%) [82]. The same group found that male APPswePS1dE9 mice, compared to non-tg littermates, did not show object recognition deficits at 5 min or 24 h after training at 5 months of age [83]. In another laboratory, male, 3.5-month-old APPswePS1dE9 mice did not differ from C57BL/6 controls in NOR performance, measured as discrimination index (index values for both genotypes around 0.5; for index, 0 equals no preference) [84]. The mice used in those three studies were younger and likely did not have substantial amyloid deposition and thus it is not incredibly surprising genotypes did not differ. However, in a study of 7.5-month-old female APPswePS1dE9 mice (males not tested), versus non-tg littermates, no effect of genotype was found on NOR recognition index scores (62 vs. 65%, respectively) [85]. These results are closest to ours, we used non-tg littermates as controls, as our mice were ~6 months of age, had average recognition index scores between 50 and 60%, and genotypes did not differ. However, some studies do show that transgenic mice perform worse. Male APPswePS1dE9 mice performed worse (lower discrimination index at 2 h) than C57BL/6 mice at 6 months of age but not at 3 months of age [49]. This same outcome was found in another study, 10-month-old male APPswePS1dE9 mice performed worse (RI ~50%) than C57BL6/J6 mice (RI ~65%) [86]. Lastly, when compared to non-tg littermates, 7.5-month-old, male APPswePS1dE9 mice performed worse on the NOR (RI Tg = ~50%, non-Tg = ~65%) [87].

A few other studies assessed the impact of high-fat diet on NOR performance in this line of mice. Among APPswePS1dE9 mice, males fed a high-fat diet performed worse on the NOR (as per RI) than males fed a standard diet at 7 months of age but not at 16 months [88]. Interestingly, in that study using only Tg mice, the RI scores were between 40 and 60%, similar to what we observed. Two other studies suggest that diet and genotype interact. Ettecho and colleagues [89] found that 3-month-old, male APPswePS1dE9 mice performed worse than C57BL/6 wildtype controls on the NOR (via discrimination index), but only if they were fed high fat (vs. control) diet. Petrov and colleagues [55] report that 6-month-old, male APPswePS1dE9 mice performed worse on the NOR (via discrimination index), compared to C57BL/6 controls, and this occurred when fed high fat or normal diet (discrimination index scores for Tg mice were ~0–0.15).

In summary, some previous studies have found that this line of AD mice performs worse on the NOR task, but others, including us, have not. Most studies use only one sex (typically male) and often have C57BL6 mice as the control, instead of non-tg littermates. Given the role of maternal effects and off-target impacts of transgenic line creation, non-tg littermates are the more appropriate control. However, source of control mice does not appear to fully drive results, as differences have been found with and without non-tg littermates as controls. Lastly, in our NOR tests, all mice appeared to struggle to recognize the familiar object, as recognition index scores were consistently between 50 and 60 (a score of 50 suggests no object preference/recognition). Mice were exposed to the familiar object during training and then again in each of the 3 tests. The final two novel objects (6 h and 24 h tests) were more distinct from the familiar object, and therefore mice might have shown an increase in recognition over time. However, the average recognition index scores did not increase over time, suggesting that repeated exposure to the familiar object (during subsequent testing) did not reinforce recall, and that object appearance did not influence behavior. However, when combined with

previous data, in this mouse model, it seems that RI scores are low and that overall, these animals do not perform exceptionally well at object discrimination (e.g., multiple studies report RIs of 40–60%).

In the MWM, performance improved over time and mice found the platform more quickly with daily practice. However, neither genotype nor diet robustly impacted outcomes in this test. Our analyses did reveal some 4-way interactions, but post hoc tests did not reveal consistent outcomes and thus these results should be treated cautiously. Among transgenic females, on day 1 (training day) of the 6-day protocol, high-fat-EPA-fed mice found the platform faster than did high-fat-fed mice. Additionally, among transgenic males, on day 2 of the 6-day protocol, high-fat-EPA-fed mice found the platform faster than did high-fat-fed mice, and on day 5, low-fat-fed mice found the platform faster than high-fat-fed mice. These results are consistent with our prediction that high-fat diet would relate to worse performance and that adding EPA would ameliorate effects. We also found that among high-fat-fed mice, on days 3 and 5 of the 6-day protocol, transgenic mice took longer to find the platform than non-transgenic mice, suggesting subtle diet and genotype interactions on behavior. However, on day 3 of the 6-day protocol, among non-transgenic males, high-fat-fed mice found the platform *faster* than low-fat- or high-fat-EPA-fed mice, which is contrary to our predictions. Although some of our results suggest diet may influence MWM performance, we found no robust main effects, and these tests should be replicated to assess consistency of findings.

Other studies of APPswePS1dE9 mice in the MWM have found conflicting results, with some supporting genotype differences in performance and some not (see Table 1 in [90]). For example, male APPswePS1dE9 mice performed worse (higher average escape latency and less time in the platform quadrant during probe) than wildtype mice at 7 months of age [91]; water temperature was 24–25°C, female not tested, housing condition (isolated or group housing) did not impact performance. In another study, APPswePS1dE9 mice performed worse than wild-type mice in the hidden platform MWM at 9.3 and 14.8 months of age, but not at 3.6 months [90]; water temperature was 25–27°C, sex not specified, and duration of MWM testing was longer than what we used. In mice aged 10–15 months, APPswePS1dE9 mice took longer to find the platform than did non-transgenic littermates [92]; water temperature was 22°C, mixed sex groups. At 13 months of age, APPswePS1dE9 mice took longer to find the platform than non-transgenic littermates [93]; water temperature was 20°C, females only. However, other studies have reported equivocal findings with some MWM outcomes differing but others not. In young mice, APPswePS1dE9 females did not differ from non-transgenic littermates in escape latency at 2.5 or 3.5 months of age, but at 3.5 months, transgenic mice spent less time in the platform quadrant during the probe trial [94]; water temperature not specified. In 9-month-old APPswePS1dE9 mice, genotype differences were only apparent during a reversal learning portion of the MWM, and only for females – mice did not differ in escape latency when the platform was in the same location across trials [95]; water temperature not specified, males and females tested, non-transgenic littermates as controls. A similar result was found in 8-month-old males, in that genotype differences were apparent in a reversal learning MWM protocol and only on the last of four trials [96]; water temperature 22°C, males only, wildtype controls. Lastly, a study on 13-month-old female mice reported no genotype differences in the hidden platform MWM task; however, all mice learned as escape latencies decreased over time [97]; water temperature 24–27°C, only females, non-transgenic littermates as controls.

It is not clear why this variation occurs, but experimental variables, such as water temperature, age, sex, background strain, and between-trial interval can matter [98–101]. Water temperature can be a major factor as cold swimming water can cause hypothermia. APPswePS1dE9 versus non-transgenic littermates, displayed greater decreases in body temperature when the MWM pool was kept at 20°C and females showed greater body temperature decreases than males [101]. In rats, hypothermia was associated with impaired MWM performance [102], if water temperature differentially and systematically impacts body temperature, this could influence outcomes. However, the above listed studies used pool temperatures from 20 to 27°C (our temperature was 25–26°C) and reported different outcomes, thus that variable does not appear to individually drive results.

Non-Cognitive Behaviors – Pain Latency and Stress Coping

Our major predictions pertaining to diet and genotype were not supported in the non-cognitive behavioral measures assessed here. We predicted that high-fat diet and genotype would both decrease pain threshold (i.e., decrease latency) but no differences among any groups were noted in the hot plate test. However, we did

find that fewer transgenic, versus non-transgenic mice, showed nociceptive behaviors (responded at all during test), suggesting that transgenic mice had a *higher* threshold for pain. In obesity, several cytokines are elevated (e.g., IL-1, IL-6, TNFα) and these same cytokines are involved in the processing of pain and can activate the HPA axis [103–106]. Increased stress and prolonged HPA axis activity are associated with obesity, inflammation, changes in immune regulators, sympathetic tone, glucose metabolism, mitochondrial dysfunction, and hyperalgesia [14, 105, 107–111], providing a connection among diet, genotype, HPA axis, and pain. Additionally, recent data from humans suggest that adiposity is a strong predictor of pain, as central obesity almost doubled the risk for chronic pain, even when controlling for insulin resistance, inflammation, and pain-related comorbidities [112]. Thus, it is likely that both pro-inflammatory cytokines and adiposity play a role in obesity-related pain. We found elevated corticosterone in our high-fat-fed mice, as predicted, but did not find a corticosterone difference by genotype. In our study, mice on the high-fat diet, especially males, exhibited elevated body mass and fat percentage [54]. We have also previously shown that dietary supplementation with EPA reduced body weight, adiposity, glucose tolerance, and insulin resistance; and reduce obesity-associated liver steatosis and inflammation as well as decrease white adipose tissue and systemic inflammation [113–117]. Thus, we predicted diet would impact pain, but did not find that here.

The FST has routinely been used as an assessment of antidepressant medication and behavioral despair in rodents [66, 118]. However, this task may more accurately be described as a stress coping task, with outcomes that may or may not be related to depressive-like behavior [119]. Forced swimming is also a physiological stressor and results in activation of the HPA axis [70, 120, 121]. Here, the FST was used to (a) assess corticosterone response to a physiological stressor, and (b) determine if experimental groups differed in their behavioral response to inescapable swimming. In male rats, prior long-term injection with corticosterone increased the duration of time animals spent immobile in the FST, and this was independent of locomotor activity or muscle strength [122, 123]; however, effects may be sex dependent as female rats showed increased duration swimming in response to chronic corticosterone administration [124]. Thus, in line with the GCH of aging and the vicious cycle of stress, and the role of high fat diet on HPA activity, we predicted transgenic mice and those fed a high-fat diet would have higher baseline corticosterone, and this would relate to more time immobile in the swim task. Our results did not support these predictions.

Overall, transgenic mice were more active (less time immobile) than non-transgenic mice. It is well known the female mice fed a high-fat diet do not gain body mass to the same extent as males, and this was true for the mice in our study [54]. When we controlled for body mass in the forced swim trials, the initial sex difference (males spending more time immobile than females) disappeared, but the effect of genotype remained. We also predicted mice fed high-fat diet would spend more time immobile than mice fed low-fat or high-fat with EPA, but this was not supported by our data (either with or without body mass as a covariate). We did find that mice fed high-fat-EPA spent more time swimming than mice on the low-fat diet. Overall, transgenic mice and those fed high-fat diet with EPA spent the most time swimming.

Activity Levels – Sex and Genotype

A consistent finding across our dataset is that females are more active than males and transgenic mice were more active than non-transgenic mice. Females covered more distance in the NOR arenas, and covered more distance, and had a higher average velocity during the overall MWM and during the MWM probe trial. Transgenics consistently covered more distance in the NOR arenas, had higher mean velocity overall in the MWM, and spent less time immobile in the forced swim. These data are consistent with energy expenditure measures on these same mice, reported in our companion manuscript [54]; when mice were tested in metabolic cages, transgenic mice had higher VO_2 consumption. Additionally, female mice had higher VO_2 consumption compared to male mice, and females covered more distance during the metabolic cage trials [54].

Previous studies using APPswePS1dE9 and APPxPS1 mice have found male and female transgenic mice are more active than non-transgenic mice in a variety of behavioral assessments across ages, e.g., open cage exploration, 2 and 4 months, males and females [125]; open field, 3.5 and 5.5 months, males [67]; novel object, 4 months, females [82]; open field and plus maze, 6 months, males [126]; light dark test, 6.5 months, males [127]; novel cage test, 12 months, males and females [128]; open field, 8–9 months, males and females [129]. Thus, the reporting of hyperactive behavior in this line of mice has been consistent, and work from Rodgers and colleagues suggests this is due to early-life overexpression of APP, a byproduct of the transgene expression in this line [125].

Overall, our data suggest male and female mice differ in corticosterone (see above) and activity level. Females also do not gain as much weight on a high-fat diet or show as robust metabolic effects [54]. Sex has more recently been recognized as an important variable in preclinical and clinical studies [36], particularly in studies addressing HPA axis function and mental health [35, 37, 76]. Gonadal steroids interact with the HPA axis, and these systems have bidirectional impacts on each other [130, 131]. Previously, researchers focused on male mice due to the (erroneous) assumption that females are more variable than males [132]. Recent studies have refuted that assumption [133, 134]. Thus, our data add to the growing body of literature on sex differences in clinically relevant physiology and behavior.

Correlational Data

In addition to exploring group differences (e.g., main effects of diet, genotype, and sex) we were interested in how variables related to one another within individual animals. Consistent with a previous study looking at relationships among hormones and behavior in APPswePS1dE9 mice [67], we did not find the predicted correlations here. If variables of diet and HPA axis function relate directly to behavioral changes, we would expect certain relationships to be present within individuals. Additionally, there are several frameworks that make predictions about early life stress and/or corticosterone and future behavioral and physiological outcomes [35, 75, 135]. Given that obesity results in alterations to HPA axis function and cognition, we expected body mass to be positively related to corticosterone and negatively related to cognitive scores. We found the opposite relationship for corticosterone (higher body mass was related to *lower* corticosterone), and body mass was not robustly related to cognitive outcomes. Additionally, using the GCH framework, we would predict that higher corticosterone would be associated with lower cognitive performance; no measure of behavior was significantly related to corticosterone. Thus, none of our predicted correlations were supposed, suggesting that on an individual level, these variables are not strongly related.

In addition to hypothesized relationships, we conducted some exploratory correlational analyses and found some noteworthy outcomes about repeatability of dependent variables. First, baseline corticosterone at 2 months of age was significantly related to baseline corticosterone at 6, 8, and 10 months, and with post-swim corticosterone at 10 months, suggesting corticosterone changes consistently within individuals over time. Additionally, distance traveled in the NOR arena at 1 h was positively correlated with distance at 6 and 24 h, again suggesting consistent performance over time within individuals. Lastly, latency to find the platform in the MWM on day 3 was significantly related to latency on days 4 and 5 of the test, thus, again, behavior is consistent within an individual over time.

Summary and Conclusions

In this 8-month longitudinal study, we tested several well-defined, a priori hypotheses about the impacts of genotype, diet, sex, and their interaction on behavioral and physiological outcomes in APPswePS1dE9 mice. These mice were part of a larger study, and in a companion manuscript we reported additional physiological impacts of diet and genotype, including serum and brain amyloid β [54] and metabolic hormones in these same mice. However, despite physiological changes in glucocorticoids (this manuscript), amyloid beta [54], and metabolic profiles [54], we did not find robust differences in behavioral outcomes (assessed in this manuscript). Our findings are consistent with other recent reports from our laboratory. In another study, we also reported that even though transgenic mice had high brain amyloid beta burden, genotype did not adversely impact cognitive performance [67, 69, 136]. Others have also noted that aged transgenic mice did not show cognitive deficits [127].

In our large study of these mice, we found, not surprisingly, that transgenic mice had higher brain and serum amyloid beta [54], and that for transgenic males, dietary supplementation of high-fat diet with EPA reduced serum amyloid beta concentrations. Additionally, EPA dietary supplementation reduced brain APP mRNA levels (Yavari et al. [54], manuscript in preparation), decreased baseline corticosterone, and decreased corticosterone response to a swim stressor (see above). However, despite the impact of diet on amyloid beta and glucocorticoid levels, we did not find impacts on cognition. Given the amyloid (cascade) hypothesis [137, 138], the GCH [16, 18], and the vicious cycle of stress [9], we would have expected a clear relationship among genotype, diet, and cognitive function, but we did not find that here, suggesting the relationship among these variables is complex.

Given the above and the recent discussion about the validity of the amyloid hypothesis [139, 140], it is possible that amyloid beta is not driving the wide-spread changes in cognitive function; that amyloid is only part of the pathology or that amyloid protein variants are critical;

and/or that environmental or developmental interactions, study design and time course, age, and experimental methodology are relevant for understanding this disease. This is not a new realization, and previous literature suggests Alzheimer's is not amyloid-centric [141] and/or may have two parts – a pre-amyloid stage and then a later amyloid-associated stage [142]. The pre-amyloid dysfunction, primarily driven by neuronal energy deficits and high oxidative stress, is likely sufficient to drive cognitive deficits, and then trigger amyloidogenic processes [142]. Thus, it is possible that amyloid beta is a downstream outcome of neuronal dysfunction, but not a causal factor. This framework is fitting with the majority of AD cases, as most are late-onset or sporadic in nature and only ~1–5% of are due to familial genetic mutations in APP and PSEN1 and 2 genes [143]. Going forward, studies with this amyloidogenic mouse model could be informative for addressing questions of when amyloid burden is necessary and sufficient to result in physiological and behavioral changes.

Recent calls for new drugs for AD have suggested targeting neuroinflammatory pathways and mitochondrial dysfunction [139]. Moreover, if, as recently proposed, AD is better characterized by dysfunction of metabolic parameters [144], then studies investigating diet, metabolic factors, behavior, glucocorticoids (a major metabolic hormone), and their associated pathways and impacts on neuronal function are needed. Studies using EPA and PUFAs are particularly promising, as these substances can improve metabolic regulation and alter mitochondrial function [113–117]. Interestingly, the newly FDA-approved AD therapies – Aducanumab (Aduhelm) and Lecanemab-irmb (LEQEMBI), monoclonal antibodies that target amyloid beta – successfully reduce amyloid in the brain but that reduction does not translate to consistent improvements in patient outcomes or cognitive function [145, 146]. This result is reminiscent of data from over 10 years ago when amyloid-targeted drugs failed clinical trials for safety or for lack of efficacy on cognitive function [142]. As current pharmaceuticals are cost prohibitive for the majority of those who suffer from dementia [147]; cheaper and more easily accessible interventions for dementia, including AD, are desperately needed [148]. Encouragingly, if future studies corroborate our findings and continually find that dietary supplementation with EPA produces amyloid-reducing effects, similar to the two currently approved therapeutic antibody drugs, dietary interventions could be an effective and easily implementable preventive approach for a broader population. In summary, our data add to the larger, pre-clinical and clinical discussions [149] of whether amyloid beta is the biomarker driving behavioral and cognitive changes seen in AD and related dementia.

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Statement of Ethics

TTU has AAALAC accreditation, and all procedures were approved by the TTU IACUC (IACUC protocol number: T19040) and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Study conceptualization, funding acquisition, experimental design, and project management: B.H., L.R., and N.M.M.; methodology development: B.H., P.L.M., K.A.M., A.C., and S.S.; genotyping: S.S.; diet maintenance and feeding: M.Y., L.R., S.S., and C.B.; ethogram development: B.H., M.Y., P.L.M., A.C., and S.T.; behavioral trial conduction: B.H., M.Y., L.P.M., K.A.M., and A.C.); scored behavioral videos: L.P.M., K.A.M., A.C., and S.T.; blood collection: B.H. and M.Y.; hormone assays, statistics, figures, and writing of original draft and revision: B.H.; reviewing and editing B.H., M.Y., L.P.M., K.A.M., A.C., S.T., S.S., C.B., and N.M.M.; data curation: B.Y., M.Y., L.R., and N.M.M.

Data Availability Statement

The data that support the findings of this study are not publicly available due to ongoing analyses from our dataset but are available from the corresponding authors B.N.H. and N.M.M. upon reasonable request. Also, see our supplemental tables for additional analysis information and output.

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