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Obesity Accelerates Alzheimer-Related Pathology in *APOE4* but Not *APOE3* Mice

Obesity increases AD in *APOE4* but not *APOE3* mice

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Abstract

Alzheimer's disease (AD) risk is modified by both genetic and environmental risk factors, which are believed to interact to cooperatively modify pathogenesis. Although numerous genetic and environmental risk factors for AD have been identified, relatively little is known about potential gene-environment interactions in regulating disease risk. The strongest genetic risk factor for late-onset AD is the ε4 allele of apolipoprotein E (*APOE*4). An important modifiable risk factor for AD is obesity, which has been shown to increase AD risk in humans and accelerate development of AD-related pathology in rodent models. Potential interactions between *APOE*4 and obesity are suggested by the literature but have not been thoroughly investigated. In the current study, we evaluated this relationship by studying the effects of diet-induced obesity in the EFAD mouse model, which combines familial AD transgenes with human *APOE*3 or *APOE*4. Male E3FAD and E4FAD mice were maintained for 12 weeks on either a control diet or a western diet high in saturated fat and sugars. We observed that metabolic outcomes of diet-induced obesity were similar in E3FAD and E4FAD mice. Importantly, our data showed a significant interaction between diet and *APOE* genotype on AD-related outcomes in which western diet was associated with robust increases in amyloid deposits, β-amyloid burden and glial activation in E4FAD but not in E3FAD mice. These findings demonstrate an important gene-environment interaction in an AD mouse model that suggests that AD risk associated with obesity is strongly influenced by *APOE* genotype.

Significance statement

*APOE*4 is the strongest genetic risk factor for Alzheimer's disease, but not all *APOE*4 carriers will develop the disease suggesting that *APOE* genotype interacts with other factors to modulate Alzheimer's risk. Here we show that diet-induced obesity interacts with *APOE*4 genotype to increase Alzheimer's-like pathology in an Alzheimer's transgenic mouse model that contains human *APOE*3 versus *APOE*4 isoforms. Interestingly, mice with *APOE*3 do not show diet-induced increases in pathology, suggesting that the adverse effects of obesity on Alzheimer's risk may be limited to *APOE*4 carriers. These findings identify an important gene-environment interaction that may have significant impact for understanding Alzheimer's risk and etiology and promoting development of targeted therapeutic approaches that incorporate both obesity and *APOE* genotype.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, the underlying causes of which are currently incompletely understood. Both genetic and environmental factors are important in determining individual risk for AD. The strongest genetic risk factor for late-onset AD is the ε4 allele of apolipoprotein E (*APOE*4) (Strittmatter et al., 1993; Liu et al., 2013). In the US, roughly 12% of the population carries the ε4 allele, but its frequency increases to ~60% in AD patients (Rebeck et al., 1993). *APOE*4 not only increases risk, but also accelerates the age of onset of AD (Corder et al., 1993; van der Flier et al., 2011). However, since homozygous carriers of *APOE*4 have a ~50% lifetime risk of AD, a significant number of *APOE*4 carriers never develop the disease (Genin et al., 2011). Thus, *APOE*4 likely interacts with other genetic and or environmental factors to drive AD risk.

A significant modifiable risk factor for dementia is obesity. Obesity has numerous adverse neural effects (Lee and Mattson, 2013) and increases the risk of dementia up to three-fold (Whitmer et al., 2008). Body mass index, a commonly used measure of obesity, has been shown to be associated with AD risk (Profenno et al., 2010) as well as with reduced brain volume in AD patients (Ho et al., 2010). Several studies indicate that obesity may be particularly problematic at midlife (Fitzpatrick et al., 2009; Profenno et al., 2010; Meng et al., 2014; Emmerzaal et al., 2015), suggesting that obesity contributes to the development of AD. Similar relationships have been observed in animal models. In particular, diet-induced obesity (DIO) accelerates AD-related pathology in mouse models of AD (Ho et al., 2004; Julien et al., 2010; Kohjima et al., 2010; Barron et al., 2013; Orr et al., 2014). Further, genetic models of obesity and type 2 diabetes exhibit features of AD-like neuropathology (Kim et al., 2009; Jung et al., 2013; Ramos-Rodriguez et al., 2013).

The extent to which *APOE*4 and obesity interact to regulate AD risk is unclear. Interestingly, *APOE*4 carriers can be more sensitive to metabolic consequences associated with

obesity (de-Andrade et al., 2000; Kypreos et al., 2009; Niu et al., 2009; Atabek et al., 2012; Zarkesh et al., 2012; Guan et al., 2013). Although some studies do not report an *APOE*4 bias in obesity-associated AD risk (Profenno and Faraone, 2008; Luchsinger et al., 2012), others have found that AD risk is increased by obesity (Peila et al., 2002; Ghebranious et al., 2011) and diets high in calories and fatty acids (Luchsinger et al., 2002) only in *APOE*4 carriers. Though the human literature suggests a gene-environment interaction between *APOE* and obesity in regulating development of AD, this question has not been addressed in experimental models. To study these relationships, we utilized EFAD transgenic mice, which combine AD transgenes with targeted replacement of mouse *APOE* with human *APOE* (Youmans et al., 2012). We compared metabolic and AD-related effects of western diet in male *APOE*3 (E3FAD) and *APOE*4 (E4FAD) mice. Here we report that diet-induced obesity increases amyloid pathology and gliosis almost exclusively in E4FAD mice. Our data reveal a gene-environment interaction between *APOE* genotype and obesity, suggesting that *APOE*4 carriers may be more susceptible to obesity associated increases in AD risk.

Materials and Methods

Animal Procedures

A colony of EFAD mice, which are heterozygous for the 5xFAD transgenes and homozygous for human *APOE*3 or *APOE*4 (Youmans et al., 2012), were maintained at vivarium facilities at [Author University] from breeder mice generously provided by Dr. Mary Jo LaDu (University of Illinois at Chicago). All animals were housed under a 12-hour light/dark cycle with lights on at 6 AM and *ad libitum* access to food and water. At 3 months of age, male E3FAD and E4FAD mice were randomized to dietary treatment groups (N = 7-11/group): control diet (10% fat, 7% sucrose; #D12450J Research Diets, Inc., NJ, USA) or western diet (45% fat, 17% sucrose; #D12451, Research Diets, Inc.). EFAD mice were maintained on experimental diets for 12 weeks, an exposure period previously established to yield obesity-induced metabolic

impairments in *APOE* mice (Arbones-Mainar et al., 2010; Segev et al., 2016). Body weight and food consumption were recorded weekly.

At the end of the treatment period, mice were anesthetized with inhalant isoflurane and transcardially perfused with ice-cold 0.1 M PBS. The brains were rapidly removed and immersion fixed for 48 h in 4% paraformaldehyde/0.1 M PBS, then stored at 4°C in 0.1 M PBS/0.3% NaN_3 until processed for immunohistochemistry. Gonadal and retroperitoneal fat pads were dissected and weighed as a measure of adiposity, and snap frozen for RNA extraction. All animal procedures were carried out under protocols approved by the [Author University] Institutional Animal Care and Use Committee and in accordance with National Institute of Health standards.

Glucose, Cholesterol, and Triglyceride Measurements

Blood glucose readings were measured after overnight fasting (16 h) every four weeks beginning at week 0 of the 12-week treatment period. Blood was collected from the lateral tail vein and immediately assessed for glucose levels using the Precision Xtra Blood Glucose and Ketone Monitoring System (Abbott Diabetes Care, CA, USA).

Glucose tolerance testing was performed at week 11. Fasting, baseline glucose readings were taken after which mice were administered a glucose bolus (2 g/kg body weight) via oral gavage. Blood glucose levels were recorded 15, 30, 60, and 120 min after the glucose bolus was given. Area under the curve was calculated.

Plasma cholesterol and triglyceride levels were enzymatically determined at the conclusion of the experiment using commercially available kits (LabAssay Triglycerides #290-63701, Wako Chemicals, VA, USA; Total Cholesterol Colorimetric Assay Kit, #K603, BioVision, CA, USA). All samples were run in duplicate according to manufacturer's instructions.

Thioflavin-S Staining and Quantification

Fixed hemi-brains were fully sectioned in the horizontal plane at 40 μm using a vibratome (Leica Biosystems, IL, USA). Every eighth section was stained for thioflavin S (#230456, Sigma-Aldrich, MO, USA) using standard methodology. Sections were mounted and allowed to dry overnight, after which they were washed three times in 50% ethanol for 5 min each, then washed in double-distilled H_2O before being incubated for 10 min in 1% thioflavin-S dissolved in H_2O. Stained slides were then rinsed in 70% ethanol before being dehydrated and coverslipped in aqueous anti-fade mounting medium (Vector Laboratories, CA, USA). Digital images were captured at 20X magnification using an Olympus BX50 microscope equipped with a DP74 camera and CellSens software (Olympus, Tokyo, Japan). The number of spherical thioflavin-positive deposits were counted using NIH ImageJ 1.50i (US National Institutes of Health, MD, USA) with the cell counter plugin to mark stained plaque-like structures. Thioflavin-positive deposits were counted in entorhinal cortex (3 fields/section), subiculum (2 fields/ section), and hippocampal subfields CA1 (3 fields/section) and CA2/3 (3 fields/section), across 4 sections per animal, for a total of ~44 fields per brain.

Immunohistochemistry

Immunohistochemistry was performed using a standard avidin/biotin peroxidase approach with ABC Vector Elite kits (Vector Laboratories). Aβ immunohistochemistry was performed on every eighth section using sections immediately adjacent to those processed for thioflavin S. Briefly, sections were pre-treated with 95% formic acid for 5 min, then rinsed in TBS before being treated with an endogenous peroxidase blocking solution for 10 min. After three 10 min washes in 0.1% Triton-X/TBS, sections were incubated for 30 min in a blocking solution consisting of 2% bovine serum albumin in TBS. Blocked sections were incubated overnight at 4°C in primary antibody directed against Aβ (#71-5800, 1:300 dilution, Invitrogen, CA, USA) that was diluted in blocking solution. Next, sections were rinsed and incubated in biotinylated

172 secondary antibody diluted in blocking solution. Immunoreactivity was visualized using 3,3'-
173 diaminobenzidine (Vector Laboratories). Additional sections were similarly immunostained
174 without formic acid pretreatment using IBA-1 (#019-19741, 1:2000 dilution, Wako) and GFAP
175 (#ab7260, 1:1,000 dilution, abcam, MA, USA).

176 To quantify the percent area occupied by Aβ immunoreactivity (Aβ load), images of non-
177 overlapping fields were taken at 20X magnification in entorhinal cortex (3 fields/section),
178 subiculum (3 fields/section), and hippocampal subfields CA1 (5 fields/section) and CA2/3 (3
179 fields/section) across 4 tissue sections, for a total of ~56 images per brain. Images were digitally
180 captured using an Olympus BX50 microscope and DP74 camera paired with a computer
181 running CellSens software (Olympus). The pictures were converted to grayscale images and
182 thresholded using NIH ImageJ 1.50i to yield binary images separating positive and negative
183 immunostaining. Aβ load was calculated as the percentage of the total area that was positively
184 immunolabeled.

185 Microglia and astrocyte activation was quantified using live imaging (Olympus BX50,
186 CASTGrid software, Olympus) at 40X magnification. Each cell was categorized as either resting
187 or reactive based on its morphology, as reported in previous studies (Ayoub and Salm, 2003;
188 Wilhelmsson et al., 2006). Specifically, microglia were scored as resting (type 1) if they had
189 spherical cell bodies, with numerous thin, highly ramified processes. Cells were scored as type
190 2 cells if they exhibited enlarged rod-shaped cell bodies with fewer processes that were shorter
191 and thicker, and scored type 3 cells if they had very few or no processes or several filopodial
192 processes. Both type 2 and type 3 morphologies were considered an activated microglia
193 phenotype. Astrocytes were visualized with GFAP immunostaining and categorized as
194 exhibiting either nonreactive (normally sized cell bodies with a few rather short projections) or
195 reactive (both cell bodies projections are enlarged) morphology phenotypes. Entorhinal cortex
196 (4 fields/section), subiculum (4 fields/section), and hippocampal subfields CA-1 (5 fields/section)

and CA-2/3 (3 fields/section) were quantified for both microglia and astrocytes. The number of cells across brain regions scored for each animal averaged ~700 microglia and ~600 astrocytes.

RNA Isolation and Real-time PCR

For RNA extractions, gonadal fat pads and hippocampi were homogenized using TRIzol reagent (Invitrogen Corporation), following the manufacturer's protocol. The RNA pellet was treated with RNase-free DNase I (Epicentre, WI, USA) for 30 min at 37°C, and a phenol/chloroform extraction was performed to isolate RNA. The iScript cDNA synthesis system (Bio-Rad, CA, USA) was used to reverse transcribe cDNA from 1 μg of purified RNA. Real-time quantitative PCR was performed on the resulting cDNA using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and a Bio-Rad CFX Connect Thermocycler. All measurements were performed in duplicates. Quantification of PCR products was carried out by normalizing with a combination of corresponding hypoxanthine-guanine phosphoribosyltransferase (HPRT) and succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial (SDHA) expression levels from the gonadal fat samples, and with β-actin expression levels from hippocampus, using the ΔΔ-CT method to obtain relative mRNA levels. Gonadal fat was probed for levels of cluster of differentiation factor 68 (CD68) and EGF-like module-containing mucin-like hormone receptor-like 1 (F4/80), while hippocampus was probed for β-secretase 1 (BACE1), neprilysin, insulin degrading enzyme (IDE), CD68, glial fibrillary acidic protein (GFAP), and cluster of differentiation factor 74 (CD74). Primer pair sequences are shown in Table 1.

Statistical Analyses

For the analysis of body weight and glucose tolerance data, two-way repeated measures ANOVAs were run using the Statistical Package for Social Sciences (SPSS; version 23, IBM, IL, USA). All other data were analyzed by two-way ANOVA using Prism (Version 5, GraphPad

Software, Inc.; CA, USA). In the case of significant main effects, planned comparisons between groups of interest were made using the Bonferroni correction. All data are presented as the mean ± the standard error of the mean (SEM). Significance was set at a threshold of $p < 0.05$. Statistical results are presented in Tables 2 and 3.

Results

Obesity-related outcomes of western diet

To begin investigating whether there are gene X environment interactions between *APOE* and western diet, we first compared measures of diet-induced obesity in E3FAD versus E4FAD mice following the 12-week exposure to control and western diets. The control diet was associated with < 1% gain in body weight in both E3FAD and E4FAD mice, whereas western diet yielded a 39 ± 7.7% increase in body weight in E3FAD and a 24 ± 7.21% increase in E4FAD mice (Fig. 1A), such that the effects of diet did not vary significantly across genotypes ($p = 0.112$; Fig. 1A; Table 2). A 2 x 2 repeated measures ANOVA revealed a significant main effect of diet on body weight ($F = 10.51$, $p = 0.003$; Fig. 1A) in which western diet was associated with increased weight. *APOE* genotype did not significantly affect body weight ($p = 0.759$; Fig. 1A). Between group comparisons revealed that E3FAD mice fed a western diet weighed significantly more than E3FAD mice fed a control diet at 4, 8, and 12 weeks ($p < 0.05$). There were no statistically significant differences in body weights at any time point between control and western diet groups in E4FAD mice.

We next examined plasma levels of cholesterol and triglycerides as measures of adverse effects of western diet. We found that plasma cholesterol levels were significantly affected by neither genotype ($p = 0.103$) nor diet ($p = 0.221$), and did we not find an interaction effect ($p = 0.119$) (Fig. 1B; Table 2). Likewise, there were no effects of either genotype ($p = 0.46$) or diet ($p = 0.102$), or an interaction effect ($p = 0.179$) on plasma triglyceride levels (Fig. 1C).

Because metabolic impairments associated with obesity have been linked to adiposity, we assessed fat deposition across groups. We observed a significant interaction effect (F = 5.01, p = 0.033; Table 2), such that on the control diets, E4FAD mice had more gonadal fat than E3FADs (p = 0.027), but there was no difference between E3FAD and E4FAD mice on western diet (p = 0.230; Fig. 1D). Additionally, there was a significant main effect of diet (F = 37.04, p < 0.001) on weight of the gonadal fat pads, so that both E3FAD and E4FAD mice had increased fat pads with western diet (Fig. 1D). Parallel findings were observed in the retroperitoneal fat pads (data not shown). Because inflammation is an established hallmark of obesity, we examined gene expression of the macrophage markers CD68 and F4/80 by rtPCR in the adipose tissue. We found a significant main effect of diet on CD68 expression (F = 11.54, p = 0.003), though this effect reached statistical significance only in E3FAD but not in E4FAD mice (Fig. 1E). There was no statistically significant effect of genotype (p = 0.353), nor was there an interaction between diet and genotype (p = 0.366) on CD68 expression. Diet had a main effect on adipose F4/80 expression (F = 7.02, p = 0.015), and again, this effect reached statistical significance only in E3FAD mice (Fig. 1F). There was no statistically significant effect of genotype (p = .768), and no interaction effect (p = 0.288) on F4/80 expression (Table 2).

In addition to increasing body weight and adiposity, western diet can induce metabolic impairments including dysregulation of glucose homeostasis. When examining glucose clearance in the glucose tolerance test, we found a significant main effect of diet (F = 5.03, p = 0.033), such that both E3FAD and E4FAD mice fed a western diet were impaired at clearing glucose (Fig. 1G; Table 2). There was no main effect of genotype (p = 0.886), or interaction effect between diet and genotype (p = 0.750) on glucose clearance. We also calculated the area under the curve (AUC) for GTT, and found that there was a significant main effect of diet (F = 5.73, p = 0.023), but not of genotype (p = 0.817) on GTT AUC (Fig. 1H). However, the effect of diet failed to reach statistical significance when examined separately in E3FAD and E4FAD mice. There was no interaction between genotype and diet on GTT AUC (p = 0.737). Changes

in fasting glucose levels over the diet treatment period showed a trend towards a main effect of diet ($F = 3.84$, $p = 0.059$; Fig. 1I). There was no effect of genotype ($p = 0.371$) nor was there an interaction between diet and genotype ($p = 0.352$) on changes in glucose levels (Table 2).

Western diet increases β-amyloid deposition in E4FAD but not in E3FAD mice

The primary AD-related neuropathological change in EFAD mice at this age is accumulation of β-amyloid protein, largely in the form of extracellular deposits, many of which exhibit positive thioflavin-S (Thio-S) staining that is indicative of amyloid. Thus, to begin assessing AD-related neuropathology, Thio-S positive plaques were counted in entorhinal cortex and in subregions of the hippocampus. Visual inspection of stained sections qualitatively showed not only the expected increase in amyloid deposits in E4FAD, but also the surprising finding that western diet increased Thio-S positive plaques only in E4FAD mice (Fig. 2A). Specifically, there were significant interaction effects between genotype and diet on Thio-S positive plaques in subiculum ($F = 9.75$, $p = 0.004$; Fig. 2C), CA1 ($F = 8.41$, $p = 0.007$; Fig. 2D), and CA2/3 ($F = 7.32$, $p = 0.011$; Fig. 2E), and a non-significant trend towards an interaction in entorhinal cortex ($F = 4.09$, $p = 0.053$; Fig. 2B; Table 2). Further analyses revealed that diet significantly increased Thio-S positive plaque counts in E4FAD but not E3FAD males across all brain regions sampled ($p < 0.01$). Additionally, there was a significant main effect of genotype even in the absence of diet, such that E4FAD mice had a greater number of Thio-S positive plaques in entorhinal cortex ($F = 50.30$, $p < 0.001$; Fig. 2B), subiculum ($F = 59.40$, $p < 0.001$; Fig. 2C), CA1 ($F = 80.58$, $p < 0.001$; Fig. 2D), and CA2/3 ($F = 46.39$, $p < 0.001$; Fig. 2E), than did E3FAD mice.

As a second measure of AD-like pathology, we assessed total β-amyloid burden by immunohistochemistry. This provides a measure of complete β-amyloid, as the antibody recognizes intra- and extracellular accumulations of Aβ, even those that have not progressed to

299 Thio-S positive amyloid deposits. Results repeated the same general pattern observed with
300 Thio-S staining. That is, (i) E4FAD mice exhibit greater β-amyloid burden and, (ii) E4FAD but
301 not E3FAD mice show increased β-amyloid accumulation with western diet (Fig. 3A). We found
302 significant interaction effects between genotype and diet in entorhinal cortex (F = 4.91, p =
303 0.035; Fig. 3B) and in CA2/3 (F = 4.48, p = 0.043; Fig. 3E), but not in subiculum (F = 0.11, p =
304 0.742; Fig. 3C) or in CA1 (F = 2.71, p = 0.110; Fig. 3D; Table 2). Bonferroni post hoc tests
305 showed that western diet significantly increased Aβ load in E4FAD but not in E3FAD mice
306 across all brain regions surveyed (p < 0.05). There was a significant main effect of genotype
307 with E4FAD mice having greater Aβ load than E3FAD mice in entorhinal cortex (F = 21.38, p <
308 0.001; Fig. 3B), subiculum (F = 25.40, p < 0.001; Fig. 3C), CA1 (F = 37.66, p < 0.001; Fig. 3D),
309 and CA2/3 (F = 47.27, p < 0.001; Fig. 3E).
310
311 *Western diet increases gliosis more strongly in E4FAD than in E3FAD mice*
312 Gliosis is an important neuropathological feature of AD that is also associated with both
313 obesity and *APOE*4. To assess gliosis, we compared both the relative cell numbers and
314 morphological activation state of microglia and astrocytes across groups. We found that, in
315 comparison to E3FAD mice, E4FAD mice consistently had a higher total number of glial cells as
316 well as a higher percentage of glial cells with reactive versus resting phenotypes. Moreover, the
317 effects of diet on glial number and reactivity were stronger in E4FAD than in E3FAD mice.
318 We first examined microglia number and morphology by IBA-1 staining. Figure 4A shows
319 a resting microglial cell with thin, ramified processes (Type 1), and activated cells with rod-
320 shaped cell bodies and fewer, thicker processes (Type 2), and amoeboid cells (Type 3). We
321 found significant interactions between genotype and diet when examining the total number of
322 microglia per mm^2 in subiculum (F = 4.75, p = 0.038; Fig. 4C) and in CA1 (F = 7.97, p = 0.009;
323 Fig. 4D), with Bonferroni post hoc tests showing that western diet increased microglia number in

E4FAD but not in E3FAD mice in these brain regions ($p < 0.05$; Table 2). There were no interaction effects on microglia number in entorhinal cortex ($p = 0.316$; Fig. 4B), or in CA2/3 ($p = 0.180$; Fig. 4E). There was a significant effect of genotype on the total number of microglia per mm^2 in entorhinal cortex ($F = 9.78$, $p = 0.004$; Fig. 4B), subiculum ($F = 42.77$, $p < 0.001$; Fig. 4C), CA1 ($F = 51.42$, $p < 0.001$; Fig. 4D), and CA2/3 ($F = 21.64$, $p < 0.001$; Fig 4E), such that E4FAD mice had a greater total number of microglia across these brain regions than did E3FAD mice. However, in entorhinal cortex, the effect of genotype was significant only in animals on a western diet.

Measures of microglial reactivity showed similar results as microglial number. Significant interaction effects between genotype and diet were observed in entorhinal cortex ($F = 5.52$, $p = 0.027$; Fig. 4F), CA1 ($F = 11.58$, $p = 0.002$; Fig. 4H), and CA2/3 ($F = 32.66$, $p < 0.001$; Fig. 4I), but not in subiculum ($p = 0.480$; Fig. 4G; Table 2). Bonferroni post hoc tests revealed that western diet increased the percent of reactive microglia in entorhinal cortex, CA1, and CA2/3 of E4FAD, but not E3FAD, male mice. There was a significant main effect of genotype even in the absence of diet, such that E4FAD mice had a greater percent of reactive microglia than E3FAD mice in entorhinal cortex ($F = 109.10$, $p < 0.001$; Fig. 4F), subiculum ($F = 19.70$, $p < 0.001$; Fig. 4G), CA1 ($F = 78.70$, $p < 0.001$; Fig. 4H), and CA2/3 ($F = 165.70$, $p < 0.001$; Fig. 4I).

We next examined astrocyte number and activation by GFAP staining. Figure 5A shows examples of a nonreactive astrocyte with a normally sized soma versus a reactive phenotype with enlarged soma and projections. For the measure of astrocyte number, the effects of diet did not differ across genotype for any of the brain regions sampled (Table 2). We found significant main effects of genotype on the total number of astrocytes in subiculum ($F = 9.95$, $p = 0.004$; Fig. 5C), though this effect was only statistically significant in animals on a western diet. There was a main effect of genotype on astrocyte number in CA1 ($F = 5.88$, $p = 0.022$; Fig. 5D), but this did not reach statistical significance when examined separately in control and western diet fed animals. There was a trend towards a significant effect of genotype in entorhinal cortex ($F =$

3.82, p = 0.060; Fig. 5B), but no effect in CA2/3 (p = 0.188; Fig. 5E). Diet had significant main effects on astrocyte number in subiculum (F = 4.79, p = 0.037; Fig. 5C), and CA2/3 (F = 4.26, p = 0.048; Fig. 5E), with a trend towards a main effect in CA1 (F = 3.55, p = 0.069; Fig. 5D), though this effect did not reach statistical significance when examined separately in E3FAD and E4FAD mice in any brain region. There was no effect of diet on astrocyte number in entorhinal cortex (p = 0.593; Fig. 5B).

When examining astrocyte reactivity, we found similar trends as with microglial reactivity. That is, there was a significant interaction effect between genotype and diet on astrocyte reactivity in entorhinal cortex (F = 4.82, p = 0.036; Fig. 5F), with western diet increasing reactivity only in E4FAD mice (Table 2). There were no significant interaction effects between genotype and diet in subiculum (p = 0.989; Fig. 5G), CA1 (p = 0.160; Fig. 5H), or CA2/3 (p = 0.132; Fig. 5I). Moreover, in the absence of diet, genotype had a significant effect on astrocyte reactivity, with E4FAD mice having a greater percentage of reactive astrocytes in entorhinal cortex (F = 46.97, p < 0.001; Fig. 5F), subiculum (F = 27.72, p < 0.001; Fig. 5G), CA1 (F = 87.49, p < 0.001; Fig. 5H), and CA2/3 (F = 11.68, p = 0.002; Fig. 5I). In CA2/3 the effect of genotype was only significant in western diet fed animals. Furthermore, western diet significantly increased astrocyte reactivity in CA1 (F = 23.82, p < 0.001; Fig. 5H), and CA2/3 (F = 7.83, p = 0.009; Fig. 5I) though this effect was only significant in E4FAD mice in CA2/3. There was a non-significant trend towards an effect of diet in subiculum (F = 3.13, p = 0.088; Fig. 5G).

E4FAD mice have increased gene expression of inflammatory markers

In order to begin addressing possible mechanisms underlying the interactive effects of *APOE*4 and western diet, we examined hippocampal gene expression of several markers related to Aβ production and clearance, as well as inflammation. Overall, our results indicate that gene expression of factors involved in Aβ clearance and production are not significantly

altered by genotype or diet, and that inflammatory gene expression is increased in E4FAD mice, without being altered by western diet (Table 3).

For BACE1, relative mRNA levels did not show evidence of an interaction between the diet and *APOE* genotypes (p = 0.874), there was no significant main effect genotype (p = 0.304), but there was a non-significant trend of increased BACE1 levels with western diet (p = 0.074). Expression of the Aβ clearance factor neprilysin was not significantly affected by genotype (p = 0.902) or diet (p = 0.126), and there was no interaction between genotype and diet (p = 0.802). Likewise, gene expression of IDE was not altered by genotype (p = 0.785), diet (p = 0.955), or the interaction between genotype and diet (p = 0.489).

In assessing gene expression of inflammatory markers we found that E4FAD mice had significantly greater levels of the microglial markers CD68 (F = 10.75, p = 0.003), the astrocyte marker GFAP (F = 14.26, p < 0.001), and the innate immune marker CD74 (F = 16.98, p < 0.001), than did E3FAD mice. However, there were no significant effects of diet on levels of CD68 (p = 0.178), GFAP (p = 0.634), or CD74 (p = 0.184). Moreover, there were no significant interactions between genotype and diet on levels of CD68 (p = 0.532), GFAP (p = 0.712), or CD74 (p = 0.335).

Discussion

The goal of this study is to examine whether *APOE* genotype and obesity interact to promote AD pathogenesis. Comparing E3FAD and E4FAD mice maintained on standard versus western diets, we demonstrate a significant gene-environment interaction whereby diet-induced obesity drives AD-related pathology primarily in *APOE*4 mice. Our results are consistent with previous findings in humans (Fitzpatrick et al., 2009; Profenno et al., 2010), and confirm studies in rodent models (Ho et al., 2004; Julien et al., 2010; Kohjima et al., 2010; Barron et al., 2013) that obesity increases risk for development of AD. Similarly, our findings replicate prior rodent data (Fryer et al., 2005; Castellano et al., 2011; Youmans et al., 2012; Rodriguez et al., 2014;

401 Cacciottolo et al., 2016) that model the human observation that *APOE*4 increases the risk and
402 or accelerates the onset of AD pathology (Corder et al., 1993; Saunders et al., 1993; Strittmatter
403 et al., 1993; Morris et al., 2010; Jack et al., 2015). Importantly, our data indicate that the effects
404 of diet-induced obesity and *APOE*4 are not strictly additive. Although *APOE*4 status is
405 associated with greater AD-like pathology on both control and western diets, obesity increased
406 AD-like pathology in E4FAD but not E3FAD mice. Our finding that E3FAD mice did not show a
407 diet-induced increase in AD-related pathology is similar to null findings in some rodent models
408 of obesity (Zhang et al., 2013; Knight et al., 2014; Niedowicz et al., 2014), suggesting that
409 deleterious effects of obesity can be regulated by genetic factors besides *APOE4*. Thus, these
410 data suggest an important gene X environment interaction in which *APOE*4 carriers are more
411 susceptible to the AD-promoting effects of obesity.

412 How neural outcomes in human populations are impacted by the relationship between
413 *APOE* genotype and metabolic risk factors remains incompletely defined. Many studies simply
414 control for *APOE* genotype rather than considering its potential moderating role in the
415 relationship between obesity and AD risk (Vanhanen et al., 2006; Luchsinger et al., 2012).
416 When *APOE* status has been considered as a modulator of AD risk associated with metabolic
417 factors, the results have been mixed. In some studies, *APOE*4 carriers showed significantly
418 more cognitive impairment in association with adverse metabolic conditions including
419 atherosclerosis, peripheral vascular disease, type 2 diabetes (Haan et al., 1999), and high
420 systolic blood pressure at midlife (Peila et al., 2001). Further, levels of senile plaques and
421 neurofibrillary tangles were highest in obese men that were also *APOE*4 carriers (Peila et al.,
422 2002). However, several other studies reported that the AD risk associated with obesity and
423 metabolic syndrome is stronger in *APOE*3 carriers (Dixit et al., 2005; Leiva et al., 2005; Singh et
424 al., 2006; Profenno and Faraone, 2008).

425 An important consideration in interpreting these seemingly discordant findings is the
426 potential role of sex differences. Although the impact of sex differences in the interactions

among obesity, *APOE*, and AD risk has not been thoroughly addressed, AD is characterized by numerous sex differences (Li and Singh, 2014; Pike, 2017). Further, the AD-associated risk of *APOE*4 appears to disproportionately affect women (Payami et al., 1994; Farrer et al., 1997; Altmann et al., 2014). Additionally, there are sex differences in various aspects of obesity (Lovejoy et al., 2009; Mauvais-Jarvis, 2015; Moser and Pike, 2016), including observations that women exhibit relative protection against obesity until menopause (Meyer et al., 2011; Sugiyama and Agellon, 2012; Bloor and Symonds, 2014). Given that sex differences have been found in each of these factors, future studies should address sex as a possible mediator in the relationship between *APOE*4 and obesity. Ongoing projects in our lab have begun to address this issue using female E3FAD and E4FAD mice.

How obesity and *APOE* interact to regulate AD pathogenesis remains to be determined. One candidate mechanism linked to both factors is metabolic impairment. Obesity is strongly associated with development of impaired glucose and insulin metabolism (Kahn et al., 2006; Singla et al., 2010), which are also characteristic of AD patients and have been proposed as possible mechanisms driving AD pathogenesis (Craft, 2005; Martins et al., 2006; Craft, 2009). Notably, *APOE* genotype affects metabolic responses to diet (Snook et al., 1999; Barberger-Gateau et al., 2011), and several studies show that *APOE*4 carriers are at increased risk for a number of metabolic disturbances (de-Andrade et al., 2000; Oh and Barrett-Connor, 2001; Elosua et al., 2003; Marques-Vidal et al., 2003; Sima et al., 2007; Kypreos et al., 2009; Niu et al., 2009; Atabek et al., 2012; Zarkesh et al., 2012; Guan et al., 2013), though some studies find no effect of *APOE* genotype on metabolic outcomes (Meigs et al., 2000; Ragogna et al., 2011). Our findings suggest that E3FAD mice may be more susceptible to some metabolic effects of western diet, though E4FAD mice trend towards metabolic disturbances even in the absence of a western diet. Specifically, relative to E4FAD mice, E3FAD mice showed greater diet-induced body weight gain, gonadal fat inflammatory cytokine expression, and higher glucose levels on western diet. Conversely, E4FAD mice had higher gonadal fat pad weight and a trend towards

higher fasting glucose levels than E3FAD mice under the control diet condition. These findings are consistent with several previous reports showing that mice with human *APOE*3 gain more weight in response to a high fat diet than mice with either human *APOE*4 (Arbones-Mainar et al., 2008; Segev et al., 2016) or mouse *APOE* (Karagiannides et al., 2008). It is important to note that the western diet utilized in this study has elevated levels of saturated fats, cholesterol, and sucrose, all of which have been independently associated with increased AD-related pathology (Refolo et al., 2000; Oksman et al., 2006; Cao et al., 2007; Takechi et al., 2010). Understanding how *APOE* genotype interacts with various dietary components should be one target of future studies. Though metabolic factors may have a role in AD pathogenesis, our findings that metabolic outcomes of diet-induced obesity were greater in E3FAD than E4FAD mice argue against the possibility that metabolic impairment significantly contributes to the observed *APOE*4 bias in diet-induced increases in AD-like pathology.

There are several other mechanisms besides metabolic impairment that may contribute to the observed interactions among obesity, *APOE*, and AD-like pathology. One established consequence of obesogenic diets is pro-amyloidogenic alteration in the expression and or activity of factors that regulate generation and clearance of Aβ including BACE1, neprilysin, and IDE (Standeven et al., 2010; Maesako et al., 2012; Brandimarti et al., 2013; Wei et al., 2014; Maesako et al., 2015). Although we cannot exclude a significant role of such pathways in our observations, we did not observe that mRNA levels of BACE1, neprilysin, and IDE were significantly altered by either the simple or interactive effects of western diet and *APOE*. Another compelling candidate mechanism is neuroinflammation, which is widely implicated as a significant regulator of AD risk and development of AD pathology (Glass et al., 2010; Wyss-Coray and Rogers, 2012; Heneka et al., 2015). Notably, both obesity and *APOE*4 are associated with increased inflammation in brain and systemically. For example, obesity is linked with increased immune cell infiltration into brain (Buckman et al., 2014), as well as increased glial activation (Koga et al., 2014; Dorfman and Thaler, 2015; Douglass et al., 2017). In addition,

obesity increases inflammation in peripheral organs including adipose tissue (Weisberg et al., 2003; Zeyda and Stulnig, 2009) and liver (Park et al., 2010). *APOE*4 is also associated with greater levels of inflammation in the brain (Ophir et al., 2005; Vitek et al., 2009) and throughout the body (Colton et al., 2004; Gale et al., 2014). Moreover, stimulating innate inflammation in the presence of apoE4 increases cell death and damage in macrophages (Cash et al., 2012), and in microglia and neurons (Maezawa et al., 2006a; 2006b). In the context of AD pathology, *APOE*4 is associated with greater glial activation in EFAD mice (Rodriguez et al., 2014). Similarly, we found that both the total number and the relative level of morphological activation of microglia and astrocytes were higher in E4FAD than E3FAD mice. Further, we observed that E4FAD mice expressed significantly higher mRNA levels of glial markers than E3FAD mice under both control and western diets. These glial markers were significantly increased across several brain regions in response to diet-induced obesity in E4FAD but not E3FAD mice. Perhaps in contrast to our results, middle-aged female *APOE*4 mice showed higher levels of neuroinflammation in hippocampus under control diet but decreased neuroinflammation with high-fat diet, relative to age- and sex-matched wild-type mice (Janssen et al., 2016). Though the presence of familial AD transgenes and Aβ pathology in the EFAD model may account for these divergent findings, there may also be age and sex differences in inflammatory responses to both diet and *APOE*4. Further, because reactive astrocytes and microglia are associated with Aβ plaques, the changes in gliosis we observe with *APOE*4 and diet-induced obesity may be a consequence of, rather than a contributor to, Aβ pathology. Thus, additional research is needed to directly assess the potential mechanistic role of gliosis in the interaction between *APOE*4 and obesity in AD.

To our knowledge, this is the first experimental investigation examining the interaction between *APOE*4 and obesity in the context of AD. Interactions among genetic risk factors like *APOE*4 and environmental and modifiable lifestyle risk factors in AD have thus far not been well

studied, though there are some epidemiological studies consistent with this possibility (Dufouil et al., 2000; Hanson et al., 2013; Rajan et al., 2014; Wirth et al., 2014; Ishioka et al., 2016; Zheng and Li, 2016). Our findings suggest that *APOE* genotype affects the relationship between obesity and AD, such that *APOE*4 carriers may be more susceptible to obesity-associated risks than *APOE*3 carriers. This illustrates an important gene-environment interaction and points to the need for additional research exploring such relationships in the context of AD, as well as identifying underlying mechanisms. Additionally, these findings identify a large population that may be at increased risk of AD, but whose chance of developing the disease may be reduced by preventative lifestyle changes.

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840 **Table legends**

841

842 **Table 1**. Gene targets for the rtPCR analyses are listed with their corresponding oligonucleotide
843 sequences for the forward and reverse primers.

844

845 **Table 2**. Statistics are shown for each relevant figure and are listed by panel number and
846 description. The Kolmogorov-Smirnov test for normality was performed, with $p > 0.05$ indicating
847 a normal distribution. Statistical analyses were determined using 2 x 2 ANOVAs as described in
848 the Methods. The values for the *F* statistic, degrees of freedom (df), and significance (*p*) are
849 shown for the main effects of genotype and diet as well as interactions between genotype and
850 diet.

851

852 **Table 3.** Relative gene expression in hippocampus. Data are presented as mean fold
853 differences (±SEM) relative to E3FAD mice on a control diet. The Kolmogorov-Smirnov test for
854 normality was performed, with $p > 0.05$ indicating a normal distribution. Genes related to β-
855 amyloid production (BACE-1) and clearance (neprilysin, IDE) showed no significant changes
856 with either diet or genotype, while genes related to glial activation (CD68, GFAP, and CD74)
857 were increased in E4FAD mice on both control and western diets.

858

859

Figure legends

Figure 1. Metabolic outcomes associated with diet-induced obesity in E3FAD and E4FAD mice. **A)** Body weights in male E3FAD and E4FAD mice maintained on control (CTL) and western (WD) diets taken at baseline (week 0) and four-week intervals across the 12-week experimental period. **B)** Plasma levels of cholesterol and **C)** triglyceride levels in E3FAD and E4FAD mice on control and western diets at the end of the experimental period. **D)** Weight of the gonadal fat pads across groups. Relative mRNA expression of macrophage markers **E)** CD68 and **F)** F4/80 in gonadal fat, as determined by rtPCR. Data show fold differences relative to the E3FAD + control diet group. **G)** Glucose tolerance test showing blood glucose levels over time after a glucose bolus. **H)** Area under the curve (AUC) for the glucose tolerance test (GTT). **I)** Percent change in fasting blood glucose levels relative to baseline after 12-weeks of control or western diet. Data are presented as mean (±SEM) values; n=7-11/group. E3FAD mice are shown as circles, E4FAD mice are shown as squares; control diet groups are indicated as open symbols or bars, whereas western diet groups are filled symbols or bars. * $p < 0.05$ relative to genotype-matched mice in control diet condition. # $p < 0.05$ relative to E3FAD mice in same diet condition.

Figure 2. Accumulation of amyloidogenic deposits assessed by thioflavin-S staining in E3FAD and E4FAD mice across dietary treatments. **A)** Representative images of thioflavin-S staining in the subiculum of E3FAD and E4FAD males fed control and western diets. Scale bar = 50 µm. Numbers of thioflavin-S positive plaque numbers in E3FAD and E4FAD mice maintained on control and western diets were quantified in **B)** entorhinal cortex, and hippocampal subregions **C)** subiculum, **D)** CA1, and **E)** CA2/3. Data are presented as mean (±SEM) values; n=7-11/group. E3FAD mice are shown as circles, E4FAD mice are shown as squares; control diet groups are indicated as open symbols, and western diet groups as filled symbols. * $p < 0.05$

885 relative to genotype-matched mice in control diet condition. # $p < 0.05$ relative to E3FAD mice in
886 same diet condition.
887
888 **Figure 3.** Accumulation of β-amyloid deposits assessed by immunohistochemistry in E3FAD
889 and E4FAD mice across dietary treatments. **A)** Representative images of β-amyloid
890 immunoreactivity in entorhinal cortex and hippocampus in E3FAD and E4FAD males maintained
891 on control and western diets. Scale bar = 100 μm. β-Amyloid burden was quantified as
892 immunoreactivity load in E3FAD and E4FAD mice in control and western diets groups in **B)**
893 entorhinal cortex, and hippocampal subregions **C)** subiculum, **D)** CA1, and **E)** CA2/3. Data are
894 presented as mean (±SEM) values; n=7-11/group. E3FAD mice are shown as circles, E4FAD
895 mice are shown as squares; control diet groups are indicated as open symbols, and western
896 diet groups as filled symbols. * $p < 0.05$ relative to genotype-matched mice in control diet
897 condition. # $p < 0.05$ relative to E3FAD mice in same diet condition.
898
899 **Figure 4.** Microglia number and morphological status assessed by IBA-1 immunohistochemistry
900 in E3FAD and E4FAD mice across dietary treatments. **A)** Representative images of microglial
901 morphology associated with resting (Type 1) and reactive (Types 2, 3) phenotypes. Scale bar =
902 40 μm. **B-E)** Densities (cells/mm^2) of IBA-1 immunoreactive cells in E3FAD and E4FAD mice on
903 control and western diets were quantified in **B)** entorhinal cortex, and hippocampal subregions
904 **C)** subiculum, **D)** CA1, and **E)** CA2/3. **F-I)** Percentages of all IBA-1 immunoreactive cells scored
905 as having reactive phenotype (types 2 and 3) were quantified in **F)** entorhinal cortex, and
906 hippocampal subregions **G)** subiculum, **H)** CA1, and **I)** CA2/3. Data are presented as mean
907 (±SEM) values; n=7-11/group. E3FAD mice are shown as circles, E4FAD mice are shown as
908 squares; control diet groups are indicated as open symbols, and western diet groups as filled

symbols. * $p < 0.05$ relative to genotype-matched mice in control diet condition. # $p < 0.05$ relative to E3FAD mice in same diet condition.

Figure 5. Astrocyte number and morphological status assessed by GFAP immunohistochemistry in E3FAD and E4FAD mice across dietary treatments. **A)** Representative images of astrocyte morphology associated with resting and reactive phenotypes. Scale bar = 50 µm. **B-E)** Densities (cells/mm^2) of GFAP immunoreactive cells in E3FAD and E4FAD mice on control and western diets were quantified in **B)** entorhinal cortex, and hippocampal subregions **C)** subiculum, **D)** CA1, and **E)** CA2/3. **F-I)** Percentages of all GFAP immunoreactive cells scored as having reactive phenotype (type 2) were quantified in **F)** entorhinal cortex, and hippocampal subregions **G)** subiculum, **H)** CA1, and **I)** CA2/3. Data are presented as mean (±SEM) values; n=7-11/group. E3FAD mice are shown as circles, E4FAD mice are shown as squares; control diet groups are indicated as open symbols, and western diet groups as filled symbols. * $p < 0.05$ relative to genotype-matched mice in control diet condition. # $p < 0.05$ relative to E3FAD mice in same diet condition.

Table 1. Sequences of primers used in rtPCR analyses.

Target Gene	Sequence
Cluster of differentiation factor 68 (CD68)	Forward: 5'-TTCTGCTGTGGAAATGCAAG-3' Reverse: 5'-AGAGGGGCTGGTAGGTTGAT-3'
EGF-like module-containing mucin-like hormone receptor-like 1 (F4/80)	Forward: 5'-TGCATCTAGCAATGGACAGC-3' Reverse: 5'-GCCTTCTGGATCCATTTGAA-3'
Hypoxanthine-guanine phosphoribosyltransferase (HPRT)	Forward: 5'-AAGCTTGCTGGTGAAAAGGA-3' Reverse: 5'-TTGCGCTCATCTTAGGCTTT-3'
Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial (SDHA)	Forward: 5'-ACACAGACCTGGTGGAGACC-3' Reverse: 5'-GGATGGGCTTGGAGTAATCA-3'
Neprilysin	Forward: 5'-GAGAAAAGCCCACTTGCTTG-3' Reverse: 5'-GAAAGACAAAATGGGGCAGA-3'
β-Secretase 1 (BACE1)	Forward: 5'-TCGCTGTCTCACAGTCATCC-3' Reverse: 5'-AACAAACGGACCTTCCACTG-3'
Insulin degrading enzyme (IDE)	Forward: 5'-TGTTTCCACACACAGGCAAT-3' Reverse: 5'-ACCTGTGAAAAGCCGAGAGA-3'
Cluster of differentiation factor 74 (CD74)	Forward: 5'-CAAGTACGGCAACATGACCC-3' Reverse: 5'-GCACTTGGTCAGTACTTTAGGTG-3'
Glial fibrillary acidic protein (GFAP)	Forward: 5'-AACGACTATCGCCGCCAACTG-3' Reverse: 5'-CTCTTCCTGTTCGCGCATTTG-3'
β-Actin	Forward: 5'-AGCCATGTACGTAGCCATCC-3' Reverse: 5'-CTCTCAGCTGTGGTGGTGAA-3'

Table 2. Statistical analyses of metabolic and Alzheimer-related outcomes.

Figure	Kolmogorov-Smirnov Test for Normality (*p* value)	Statistical Significance
1A Body Weight	All groups at all time points are normally distributed ($p > 0.05$).	Genotype: $F_{1,29} = 0.10$, $p = 0.759$ Diet: $F_{1,29} = 10.51$, $p = 0.003$ Interaction: $F_{1,29} = 2.68$, $p = 0.112$
1B Plasma Cholesterol	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype: $F_{1,29} = 2.86$, $p = 0.103$ Diet: $F_{1,29} = 1.58$, $p = 0.221$ Interaction: $F_{1,29} = 2.60$, $p = 0.119$
1C Plasma Triglycerides	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype: $F_{1,29} = 0.56$, $p = 0.46$ Diet: $F_{1,29} = 2.87$, $p = 0.102$ Interaction: $F_{1,29} = 1.91$, $p = 0.179$
1D Gonadal Fat Weight	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype: $F_{1,29} = 0.18$, $p = 0.673$ Diet: $F_{1,29} = 37.04$, $p < 0.001$ Interaction: $F_{1,29} = 5.01$, $p = 0.033$
1E CD68	E3FAD CTL N/A E3FAD WD > 0.10 E4FAD CTL = 0.004 E4FAD WD > 0.10	Genotype: $F_{1,21} = 0.90$, $p = 0.353$ Diet: $F_{1,21} = 11.54$, $p = 0.003$ Interaction: $F_{1,21} = 0.85$, $p = 0.366$
1F F4/80	E3FAD CTL N/A E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype: $F_{1,21} = .09$, $p = .768$ Diet: $F_{1,21} = 7.02$, $p = 0.015$ Interaction: $F_{1,21} = 1.19$, $p = 0.288$
1G Glucose (GTT)	All groups at all time points are normally distributed ($p > 0.05$), except: E4FAD CTL 0 min = 0.002 E4FADWD 15 min = 0.025 E3FAD WD 30 min = 0.011 E4FAD WD 30 min = 0.008	Genotype: $F_{1,29} = 0.02$, $p = 0.886$ Diet: $F_{1,29} = 5.03$, $p = 0.033$ Interaction: $F_{1,29} = 0.10$, $p = 0.750$
1H GTT AUC	E3FAD CTL = 0.07 E3FAD WD = 0.097 E4FAD CTL > 0.10 E4FAD WD = 0.033	Genotype: $F_{1,29} = .06$, $p = 0.817$ Diet: $F_{1,29} = 5.73$, $p = 0.023$ Interaction: $F_{1,29} = 0.12$, $p = 0.737$
1I Percent Glucose Change	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype: $F_{1,29} = .83$, $p = 0.371$ Diet: $F_{1,29} = 3.84$, $p = 0.059$ Interaction: $F_{1,29} = 0.90$, $p = 0.352$
2B Thio-S: Entorhinal Cortex	E3FAD CTL > 0.10 E3FAD WD = 0.049 E4FAD CTL > 0.10	Genotype: $F_{1,29} = 50.30$, $p < 0.001$ Diet: $F_{1,29} = 6.62$, $p = 0.016$ Interaction: $F_{1,29} = 4.09$, $p = 0.053$

	E4FAD WD > 0.10	
2C Thio-S: Subiculum	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype: $F_{1,29} = 59.40$, $p < 0.001$ Diet: $F_{1,29} = 2.98$, $p = 0.095$ Interaction: $F_{1,29} = 9.75$, $p = 0.004$
2D Thio-S: CA1	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype: $F_{1,29} = 80.58$, $p < 0.001$ Diet: $F_{1,29} = 4.95$, $p = 0.034$ Interaction: $F_{1,29} = 8.41$, $p = 0.007$
2E Thio-S: CA2/3	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,29} = 46.39$, $p < 0.001$ Diet: $F_{1,29} = 7.41$, $p = 0.011$ Interaction: $F_{1,29} = 7.32$, $p = 0.011$
3B Aβ Load: Entorhinal Cortex	E3FAD CTL > 0.10 E3FAD WD = 0.002 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,29} = 21.38$, $p < 0.001$ Diet: $F_{1,29} = 7.83$, $p = 0.009$ Interaction: $F_{1,29} = 4.91$, $p = 0.035$
3C Aβ Load: Subiculum	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,29} = 25.40$, $p < 0.001$ Diet: $F_{1,29} = 11.19$, $p = 0.002$ Interaction: $F_{1,29} = 0.11$, $p = 0.742$
3D Aβ Load: CA1	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD = 0.036	Genotype $F_{1,29} = 37.66$, $p < 0.001$ Diet: $F_{1,29} = 2.91$, $p = 0.099$ Interaction: $F_{1,29} = 2.71$, $p = 0.110$
3E Aβ Load: CA2/3	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,29} = 47.27$, $p < 0.001$ Diet: $F_{1,29} = 10.36$, $p = 0.003$ Interaction: $F_{1,29} = 4.48$, $p = 0.043$
4B Microglia Number: Entorhinal Cortex	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,27} = 9.78$, $p = 0.004$ Diet: $F_{1,27} = 2.31$, $p = 0.141$ Interaction: $F_{1,27} = 1.05$, $p = 0.316$
4C Microglia Number: Subiculum	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,27} = 42.77$, $p < 0.001$ Diet: $F_{1,27} = 4.20$, $p = 0.050$ Interaction: $F_{1,27} = 4.75$, $p = 0.038$
4D Microglia Number: CA1	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,27} = 51.42$, $p < 0.001$ Diet: $F_{1,27} = 10.78$, $p = 0.003$ Interaction: $F_{1,27} = 7.97$, $p = 0.009$
4E Microglia Number: CA2/3	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,27} = 21.64$, $p < 0.001$ Diet: $F_{1,27} = 1.97$, $p = 0.172$ Interaction: $F_{1,27} = 1.90$, $p = 0.180$
4F Microglia Reactivity: Entorhinal Cortex	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,27} = 109.10$, $p < 0.001$ Diet: $F_{1,27} = 1.64$, $p = 0.212$ Interaction: $F_{1,27} = 5.52$, $p = 0.027$
4G	E3FAD CTL > 0.10	Genotype $F_{1,27} = 19.70$, $p < 0.001$

Microglial Reactivity: Subiculum	E3FAD WD > 0.10 E4FAD CTL = 0.07 E4FAD WD < 0.001	Diet: $F_{1,27} = 0.00$, $p = 0.995$ Interaction: $F_{1,27} = 0.51$, $p = 0.480$
4H Microglial Reactivity: CA1	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD = 0.04	Genotype $F_{1,27} = 78.70$, $p < 0.001$ Diet: $F_{1,27} = 5.00$, $p = 0.034$ Interaction: $F_{1,27} = 11.58$, $p = 0.002$
4I Microglial Reactivity: CA2/3	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,27} = 165.70$, $p < 0.001$ Diet: $F_{1,27} = 21.04$, $p < 0.001$ Interaction: $F_{1,27} = 32.66$, $p < 0.001$
5B Astrocyte Number: Entorhinal Cortex	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,29} = 3.82$, $p = 0.060$ Diet: $F_{1,29} = 0.29$, $p = 0.593$ Interaction: $F_{1,29} = 0.41$, $p = 0.528$
5C Astrocyte Number: Subiculum	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,29} = 9.95$, $p = 0.004$ Diet: $F_{1,29} = 4.79$, $p = 0.037$ Interaction: $F_{1,29} = 1.04$, $p = 0.316$
5D Astrocyte Number: CA1	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,29} = 5.88$, $p = 0.022$ Diet: $F_{1,29} = 3.55$, $p = 0.069$ Interaction: $F_{1,29} = 0.49$, $p = 0.489$
5E Astrocyte Number: CA2/3	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,29} = 1.82$, $p = 0.188$ Diet: $F_{1,29} = 4.26$, $p = 0.048$ Interaction: $F_{1,29} = 0.02$, $p = 0.894$
5F Astrocyte Reactivity: Entorhinal Cortex	E3FAD CTL > 0.10 E3FAD WD = 0.004 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,29} = 46.97$, $p < 0.001$ Diet: $F_{1,29} = 5.75$, $p = 0.023$ Interaction: $F_{1,29} = 4.82$, $p = 0.036$
5G Astrocyte Reactivity: Subiculum	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL = 0.045 E4FAD WD > 0.10	Genotype $F_{1,29} = 27.72$, $p < 0.001$ Diet: $F_{1,29} = 3.13$, $p = 0.088$ Interaction: $F_{1,29} = 0.00$, $p = 0.989$
5H Astrocyte Reactivity: CA1	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,29} = 87.49$, $p < 0.001$ Diet: $F_{1,29} = 23.82$, $p < 0.001$ Interaction: $F_{1,29} = 2.08$, $p = 0.160$
5I Astrocyte Reactivity: CA2/3	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype $F_{1,29} = 11.68$, $p = 0.002$ Diet: $F_{1,29} = 7.83$, $p = 0.009$ Interaction: $F_{1,29} = 2.405$, $p = 0.132$

Table 3. Relative mRNA expression of genes related to β-amyloid and glial activation.

Gene	Mean ± SEM	Kolmogorov-Smirnov Test for Normality (*p* value)	Statistical Significance
BACE1	E3FAD CTL = 1 ± N/A E3FAD WD = 1.53 ± 0.31 E4FAD CTL = 1.32 ± 0.19 E4FAD WD = 1.76 ± 0.41	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype: $F_{1,28} = 1.10$, $p = 0.304$ Diet: $F_{1,28} = 3.44$, $p = 0.074$ Interaction: $F_{1,28} = 0.03$, $p = 0.874$
Neprilysin	E3FAD CTL = 1 ± N/A E3FAD WD = 1.61 ± 0.79 E4FAD CTL = 0.94 ± 0.30 E4FAD WD = 1.79 ± 0.63	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype: $F_{1,28} = 0.02$, $p = 0.902$ Diet: $F_{1,28} = 2.49$, $p = 0.126$ Interaction: $F_{1,28} = 0.06$, $p = 0.802$
IDE	E3FAD CTL = 1 ± N/A E3FAD WD = 1.27 ± 0.39 E4FAD CTL = 1.30 ± 0.39 E4FAD WD = 1.12 ± 0.35	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL = 0.01 E4FAD WD > 0.10	Genotype: $F_{1,28} = 0.08$, $p = 0.785$ Diet: $F_{1,28} = 0.00$, $p = 0.955$ Interaction: $F_{1,28} = 0.49$, $p = 0.489$
CD68	E3FAD CTL = 1 ± N/A E3FAD WD = 1.21 ± 0.29 E4FAD CTL = 1.74 ± 0.30 E4FAD WD = 2.30 ± 0.29	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype: $F_{1,28} = 10.75$, $p = 0.003$ Diet: $F_{1,28} = 1.91$, $p = 0.178$ Interaction: $F_{1,28} = 0.40$, $p = 0.532$
GFAP	E3FAD CTL = 1 ± N/A E3FAD WD = 1.02 ± 0.11 E4FAD CTL = 1.56 ± 0.21 E4FAD WD = 2.70 ± 0.04	E3FAD CTL > 0.10 E3FAD WD > 0.10 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype: $F_{1,28} = 14.26$, $p < 0.001$ Diet: $F_{1,28} = 0.23$, $p = 0.634$ Interaction: $F_{1,28} = 0.14$, $p = 0.712$
CD74	E3FAD CTL = 1 ± N/A E3FAD WD = 1.28 ± 0.28 E4FAD CTL = 3.32 ± 0.62 E4FAD WD = 5.04 ± 1.30	E3FAD CTL > 0.10 E3FAD WD = 0.01 E4FAD CTL > 0.10 E4FAD WD > 0.10	Genotype: $F_{1,28} = 16.98$, $p < 0.001$ Diet: $F_{1,28} = 1.86$, $p = 0.184$ Interaction: $F_{1,28} = 0.96$, $p = 0.335$