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APOE4 homozygote females are resistant to the beneficial effects of 17β-estradiol on memory and CA1 dendritic spine density in the EFAD mouse model of Alzheimer's disease

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Abstract

Female *APOE4* carriers are at greatest risk of Alzheimer's disease (AD). The potent estrogen 17β-estradiol (E_2) may mediate AD risk, as the onset of memory decline coincides with the menopausal transition. Whether *APOE* genotype mediates E_2's effects on memory and neuronal morphology is poorly understood. We used the *APOE*$^{+/+}$/5xFAD$^{+/-}$ (EFAD) mouse model to examine how *APOE3* homozygote (E3FAD), *APOE3/4* heterozygote (E3/4FAD), and *APOE4* homozygote (E4FAD) genotypes modulate effects of E_2 on object and spatial memory consolidation, dendritic spine density, and dorsal hippocampal estrogen receptor expression in 6-month-old ovariectomized EFAD mice. Dorsal hippocampal E_2 infusion enhanced memory consolidation and increased CA1 apical spine density in E3FAD and E3/4FAD, but not E4FAD, mice. CA1 basal mushroom spines were also increased by E_2 in E3FADs. E4FAD mice exhibited reduced CA1 and mPFC basal spine density, and increased dorsal hippocampal ERα protein, independent of E_2. Overall, E_2 benefitted hippocampal memory and structural plasticity in females bearing one or no *APOE4* allele, whereas two *APOE4* alleles impeded the memory-enhancing and spinogenic effects of E_2.

Keywords

Alzheimer's disease; apolipoprotein E; *APOE4*; 17β-estradiol; spatial memory; object recognition; hippocampus; medial prefrontal cortex

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Declarations of Interest

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1. Introduction

Alzheimer's disease (AD), the most common form of dementia, poses a monumental global health crisis. Despite efforts by researchers to identify pharmacological treatments and drug targets, there are few disease-modifying therapies that mitigate the devastating impact of AD. Other than aging, risk factors for AD include *APOE4* genotype and female sex. *APOE4* genotype renders individuals more susceptible to AD than other *APOE* genotypes, increasing risk by as much as 2-fold in the presence of a single *APOE4* allele (Bertram, 2009; Roses, 1996). Women, especially *APOE4* carriers, are at significantly greater risk for developing AD compared to men (Altmann et al., 2014; Bretsky et al., 1999), making the examination of factors contributing to this increased risk in women critical for AD research.

One potential mediator of heightened risk for AD in women is the loss of circulating estrogens coincident with the onset of the menopausal transition. Multiple studies implicate the precipitous loss of 17β-estradiol (E_2), the most prevalent circulating estrogen, at menopause as a contributing factor for age and disease-associated cognitive decline (Jacobs et al., 2016; Paganini-Hill and Henderson, 1996). In fact, female AD patients have lower levels of endogenous E_2 than age and sex-matched controls (Manly et al., 2000; Tsolaki et al., 2005). However, clinical trials of estrogen therapy paint a conflicting portrait of its effects on AD risk; early studies offered support for the idea that E_2 may reduce risk of AD (Baldereschi et al., 1998; Joffe et al., 2006; Kawas et al., 1997; Tang et al., 1996), whereas other studies and more recent clinical trials do not (Gleason et al., 2015; Rapp et al., 2003; Shumaker et al., 2003). These discrepant reports may be related to key factors influencing estrogen therapy efficacy including age at initiation, specific estrogen treatment regimen (e.g., estradiol vs. conjugated equine estrogens), dose, length of treatment, and physical health (Henderson, 2006; Paganini-Hill and Henderson, 1996). Another critical element of cognitive responsiveness to estrogen therapy is *APOE* status. Importantly, cognition in women *APOE4* carriers appears to benefit less from estrogen therapy than in their *APOE4*-negative counterparts (Yaffe et al., 2000). This clinical work is consistent with evidence from basic research; *APOE2* and *APOE3*, but not *APOE4*, act synergistically with E_2 to promote neurite outgrowth in mixed cell cultures, suggesting that *APOE4* expression renders cells uniquely nonresponsive to E_2 (Nathan et al., 2004). Combined, the above results suggest that E_2 may be beneficial for carriers of *APOE3* alleles, and unhelpful or even deleterious for *APOE4* carriers.

In adult female mice, treatment with exogenous E_2 facilitates memory via rapid activation of cell signaling within the dorsal hippocampus (DH), which is accompanied by an increase in dendritic spine density in the DH and in the medial prefrontal cortex (Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2013; Tuscher et al., 2016a). The spinogenic effects of E_2 have been linked to improvements in learning and memory (Inagaki et al., 2012; Phan et al., 2015; Smith et al., 2009), suggesting that spinogenesis may be used as a morphological biomarker of the pro-cognitive effects of E_2. Importantly, spine density loss in the hippocampus and cortex is an established correlate of cognitive decline in AD patients, and in some cases is associated more strongly with aberrant memory function than other pathological hallmarks of AD (DeKosky and Scheff, 1990; Selkoe, 2002). Unsurprisingly, *APOE4* has deleterious effects on spine density relative to *APOE3* in both

transgenic mice and in humans, which may drive, or be a driver of, accelerated disease progression (Dumanis et al., 2009; Ji et al., 2003). Given that synaptic integrity and dendritic spine density are closely linked to learning and memory, spinogenic compounds, such as E_2, may be of benefit to AD patients. However, whether the spinogenic or mnemonic benefits of E_2 are modulated by *APOE* genotype is unknown.

The present study was designed to test whether *APOE* genotype interacts with E_2 to modulate multiple forms of memory, dendritic spine density in the DH and medial prefrontal cortex (mPFC), and DH cell signaling and estrogen receptor expression in the well-characterized EFAD mouse model of Alzheimer's disease (Liu et al., 2015; Tai et al., 2017; Youmans et al., 2012). Female EFAD-transgenic (Tg) mice ($APOE^{+/+}$/5xFAD$^{+/-}$) express 5 familial AD (FAD) mutations (5xFAD), and human *APOE3* ($APOE^{3/3}$/5xFAD$^{+/-}$; E3FAD), *APOE3/4* ($APOE^{3/4}$/5xFAD$^{+/-}$; E3/4FAD), or *APOE4* ($APOE^{4/4}$/5xFAD$^{+/-}$; E4FAD). At 6 months of age, the hippocampus and frontal cortex of E4FAD mice express striking AD-like pathology, with significantly higher accumulation of total Aβ42, soluble Aβ42, soluble oligomeric Aβ42, amyloid plaques, and total APOE relative to E3FAD mice (Youmans et al., 2012). Thus, this model allows examination of the influence of *APOE* genotype on the cognitive and neural response to E_2 against a background of AD-like pathology, which makes the EFAD model ideal for this study. First, ovariectomized female E3FAD, E3/4FAD, and E4FAD mice received bilateral dorsal hippocampal (DH) infusion of E_2 immediately after training in object recognition (OR) and object placement (OP) tasks. Given the vulnerability of the DH and mPFC to excitatory synapse loss in AD, we next quantified dendritic spine density in the CA1 region of the DH and the mPFC. Because previous studies in wild-type mice showed that E_2 rapidly increases activation of estrogen receptors and numerous cell-signaling kinases in the DH (e.g., Boulware et al., 2013; Kim et al., 2016, 2019), we also used western blotting to assess DH levels of kinase activity, estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and G-protein-coupled estrogen receptor (GPER). We hypothesized that E3FADs would be the most responsive to E_2, such that DH E_2 infusion would promote memory consolidation in both tasks and would increase dendritic spine density in both the DH and mPFC. We also hypothesized that E4FADs would be the least responsive to E_2, and that E_2's efficacy in E3/4FADs would be intermediate between E3FADs and E4FADs. Based on our previous study of sex differences in E3FAD and E4FAD mice (Taxier et al., 2022a), we expected estrogen receptor alpha (ERα) levels to be highest in the DH of E4FAD mice but predicted effects on other estrogen receptors were unclear based on a lack of previous data on expression of these receptors in EFAD mice. We also anticipated that mnemonic benefits of E_2 in EFADs, if present, could be linked to rapid activation of cell signaling. DH infusion of E_2 facilitated memory consolidation and an increase in CA1 apical dendritic spine density in ovariectomized E3FAD and E3/4FAD, but not E4FAD, mice. Thus, in mice bearing significant AD-like pathology, E_2 benefitted memory and structural plasticity in mice with zero or one copy of *APOE4*, whereas two copies of *APOE4* blocked the memory-enhancing and spinogenic effects of E_2. Understanding how *APOE4* impedes the actions of E_2 may provide new insights into the variable effects of E_2 on AD risk in women.

2. Methods

2.1. Subjects

APOE-TR$^{+/+}$/5xFAD$^{+/-}$ (EFAD) mice express five familial AD mutations (APP K670N/M671L, I716V, V717I, PS1 M146L, and L286V) under control of the neuron-specific mouse Thy-1 promoter, and express human *APOE3* or *APOE4* (Tai et al., 2017; Youmans et al., 2012). Female mice for the present experiment were homozygous for human *APOE3* (E3FAD) or *APOE4* (E4FAD), or had one copy each of *APOE3* and *APOE4* (E3/4FAD). EFAD mice were bred, weaned, and genotyped at the University of Illinois Chicago (UIC; Animal use protocol 17–066) and shipped to the University of Wisconsin-Milwaukee (UWM; Animal use protocol 19–20-03) at 2 months of age, where they were aged to 6 months before the start of behavioral testing. Prior to surgery, mice were housed in groups of up to 5 per cage and were singly housed following cannulation and ovariectomy. Mice were behaviorally tested in four separate cohorts, whereas brain analyses for all mice were conducted at the same time. The first two behavioral cohorts consisted of only E3FAD and E4FAD genotypes because E3/4FAD mice were not available at that time. When sufficient numbers of E3/4FAD mice could be generated for this project, they were added to the latter two cohorts, which included mice from all three genotypes. Thus, Ns for the E3/4FAD groups are smaller than those of the E3FAD and E4FAD groups. A subset of brains was dissected and frozen for Western blotting and the rest were Golgi impregnated for spine density analyses. Mice were maintained on a 12 h light/dark cycle with *ad libitum* access to food and water for the duration of the study. Protocols and procedures followed the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee.

2.2. Surgeries

At the start of surgery, mice received a 5 mg/kg dose of subcutaneous Rimadyl for pain management. They were then anesthetized with isoflurane in 100% oxygen (5% for induction, 2% for maintenance) and placed in a stereotaxic apparatus (Kopf instruments) for ovariectomy and cannula implantation, which occurred within the same surgical session (Lewis et al., 2008; Taxier et al., 2019). Stainless steel bilateral guide cannulae (C232GC, 22 gauge; Plastics One) were implanted into the DH (−1.7 mm AP, ± 1.5 mm ML, and −2.3 mm DV) and affixed to the skull using dental cement (Darby Dental). Dummy cannulae (C232DC; Plastics One) were used to prevent clogging of the guide cannulae. Mice received MediGel carprofen (ClearH20) for postsurgical analgesia and were given one week to recover prior to the start of behavioral testing.

2.3. Drugs and Infusions

Hydroxypropyl-β-cyclodextrin (HBC)-encapsulated E_2 (Sigma-Aldrich) was dissolved in 0.9% sterile saline to a concentration of 10 μg/μl and was infused at a rate of 0.5 μl/min for 1 min/hemisphere (Fortress et al., 2013; Kim et al., 2019; Taxier et al., 2019). Vehicle-treated mice received HBC (Sigma-Aldrich) dissolved in saline to the same concentration of cyclodextrin as in the E_2 solution. For behavioral experiments, mice received a bilateral DH infusion of vehicle or E_2 immediately post-training. Two weeks separated bouts of behavioral training to ensure that any acute effects of post-training infusions had dissipated

prior to the next infusion. Two weeks following the conclusion of behavioral testing, mice were reinfused with the same treatment they received previously, and tissue was collected 5 min later for Western blotting or 2 h later for Golgi impregnation and spine counting.

2.4. Behavioral Tasks

Object recognition (OR) and object placement (OP) were conducted as described previously (Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2013; Kim et al., 2019; Taxier et al., 2019; Tuscher et al., 2018). Mice were handled for 30 s/day for three days prior to exposure to the behavioral arena. On the second day of handling, a Lego Duplo block (6.3 × 3.1 × 2.3 cm) was placed into each home cage to acclimate mice to objects for the remainder of handling and habituation. After handling was completed, mice were allowed to habituate to the empty behavioral arena (60 cm x 60 cm x 47 cm) for 5 min/day for two days. Light levels in the area were measured daily to ensure even illumination of 75 ± 5 lux in each corner of the arena. The following day, mice were acclimated to the empty arena for 2 min, removed to a holding cage, and immediately returned to the arena and exposed to two identical objects placed 5 cm from the upper left and right corners of the arena. Mice were then allowed to freely explore the objects until they accumulated 30 s of object exploration (or until 20 min had elapsed), defined as any time a mouse's nose was within 2 cm of the object. The 30 s criterion was chosen to ensure that mice spent an equivalent amount of time exploring the training objects prior to drug infusion, as different amounts of time spent with these objects could alter the strength of the memory in such a way as to occlude effects of drug manipulation. Mice that did not reach the 30 s criterion within the allotted 20 min were excluded. Immediately following training, mice received bilateral DH infusion of HBC vehicle or E_2 and were then returned to their home cage.

OR testing was conducted 24 h after drug infusion, at which point mice were returned to the arena in which one familiar object from the training phase was replaced by a novel object. Mice were again allowed to accumulate 30 s of object exploration. OP testing was conducted 4 h after drug infusion; here, mice were allowed to explore one familiar training object in its original location, and the second training object in a new location (bottom left or right corner of the arena). Mice that remember the identity and location of the training objects, respectively, spend more time than chance (15 s) exploring the novel or moved objects during testing (Kim et al., 2019; Taxier et al., 2019; Tuscher et al., 2016b). Time spent with the objects and time to accumulate 30 s of exploration were recorded using ANYmaze automated tracking software and scored by a researcher blinded to treatment and genotype (San Diego Instruments). The order of OR and OP testing was counterbalanced across groups, and approximately two weeks separated OR training and testing from OP training and testing to allow for acute effects of post-training E_2 infusion to dissipate prior to the next training session.

Although vehicle-treated ovariectomized wild-type mice typically remember the identity and location of the training objects after a 24 h (OR) or 4 h (OP) delay, respectively (Kim et al., 2019; Taxier et al., 2019; Tuscher et al., 2016b), we have previously shown that only male E3FADs, and not female E3FADs or E4FADs of either sex, remember the training object identity or location when tested at these same delays (Taxier et al., 2022a). Thus, given

that female E3FADs and E4FADs exhibit impaired memory at the 24 h (OR) and 4 h (OP) delays, mice in the present experiment were tested using these delays to assess whether DH E_2 infusion could facilitate memory consolidation.

2.5. Western Blotting

Two weeks after the conclusion of behavioral testing, a subset of mice (10–12/group) were infused into the DH with vehicle or E_2. Mice were cervically dislocated and decapitated 5 min later, based on previous studies in wild-type mice showing that E_2 or GPER activation increases phosphorylation of numerous cell-signaling kinases, including ERK and JNK, in the DH at this time point (e.g., Boulware et al., 2013; Kim et al., 2016, 2019). Brains from each group were extracted, and the DH was bilaterally dissected immediately on ice and frozen at −80°C. Western blotting was conducted as described previously (Boulware et al., 2013; Fernandez et al., 2008; Kim et al., 2019; Koss et al., 2018; Taxier et al., 2019). Tissue samples were resuspended 1:25 weight/volume in lysis buffer containing PMSF and a protease inhibitor cocktail (Sigma-Aldrich) and homogenized using a probe sonicator (Branson Sonifier 250). Homogenates were electrophoresed on 10% TGX (Tris-Glycine eXtended) stain-free precast gels (Bio-Rad) and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) using the TransBlot Turbo system (Bio-Rad). Total protein transfer was then verified using a ChemiDoc MP gel imager (Bio-Rad).

Membranes were blocked in 5% milk and incubated with following primary antibodies overnight at 4°C: phosphorylated 42/44 ERK (#9101, 1:2,000, Cell Signaling Technology), total ERK (#9102, 1:20,000, Cell Signaling Technology), phosphorylated 46/54 JNK (#4668, 1:1000, Cell Signaling Technology), total JNK (#9252, 1:1000, Cell Signaling Technology), ERα (H-184, 1:1000, Santa Cruz Biotechnology), ERβ (#PA1-310B, 0.25μg/mL, Thermo Fisher), and GPER (ab39742, 1:250, Abcam). The following day, all blots were incubated at room temperature with a rabbit (#7074, 1:5000, Cell Signaling Technology) or mouse (#7076, 1:5000, Cell Signaling Technology) HRP-conjugated secondary antibody. Blots were then developed using Clarity Max chemiluminescent substrate (Bio-Rad) and protein expression was detected using a ChemiDoc MP gel imager (Bio-Rad). Densitometry was performed using Image Lab software (Bio-Rad Image Lab v 5.2). Blots were stripped and reprobed for β-Actin (#4967, 1:1000, Cell Signaling Technology) for protein normalization. Data were expressed as average volume intensity as a percentage compared to vehicle-treated E3FAD females.

2.6. Golgi Impregnation and Spine Counting

Whole brains (n=4–9/group) were collected for Golgi impregnation two weeks after the conclusion of behavioral testing. Mice were cervically dislocated and decapitated 2 h following DH infusion of vehicle or E_2. The 2 h time point was chosen because previous work from our lab and others indicates that DH infusion of E_2 increases DH and mPFC dendritic spine density at this delay (Murakami et al., 2006; Tuscher et al., 2016a). Golgi staining was performed as described previously (Frankfurt et al., 2011; Kim et al., 2019; Tuscher et al., 2016a) using the Rapid Golgi Stain Kit (FD Neuro Technologies). A cryostat was used to slice tissue into 100 μm sections, which were directly mounted onto gelatin-coated microscope slides. Mounted tissue was then stained according to the Rapid Golgi

Stain Kit instructions, and coverslipped with Permount. Slides were coded so that the individual counting spines was blind to treatment, and slides were kept in the dark when not in use.

Secondary basal dendrites and tertiary apical dendrites were counted from pyramidal neurons in the dorsal hippocampal CA1 and layer II/III of the prelimbic/infralimbic mPFC under an Olympus BX51WI microscope (100x with oil) using NeuroLucida (v 11.08, MBF Bioscience). Accuracy of DH cannula placement was visually validated by examining sections containing DH tissue. Dendritic segments selected for spine counting were between 10–20 μm in length and 0.5–1.3 μm thick. Neurons selected for analysis were required to have well impregnated cell bodies and dendrites, and to be clearly distinguishable from adjacent cells.

Dendritic spines were identified by an experimenter scrolling through the z-plane of stained tissue in real time, and spines were reconstructed using NeuroLucida morphometric markers. Spines were classified according to three categories based on shape: mushroom, thin, or stubby (Harris et al., 1992; Kim et al., 2019). Mushroom spines had head diameters at least twice the size of their neck diameters, whereas thin spines had head diameters less than or equal to their neck diameters. Stubby spines had neck diameters relatively equal to the total length of the spine, with no discernable spine head. Two dendritic segments/neuron and 6 cells/region were included in the analysis. After segment tracing and spine marking, data were exported to NeuroLucida Explorer (MBF Bioscience), where a Branched Structure Analysis allowed for visualization of the number and type of spines on each traced dendritic segment. Spine density was calculated as the number of spines/10 μm dendrite.

2.7. Data Analysis

All statistical analyses were conducted using GraphPad Prism 9 software (La Jolla, CA). To assess within-group learning for OR and OP, one-sample *t*-tests were used to determine whether the time spent with each object during testing significantly differed from chance (15 s). Differences in memory between groups were assessed using two-way ANOVAs with treatment and genotype as between-subject variables. Similar two-way ANOVAs were used to analyze western blot and dendritic spine density data. Significant main effects were followed by Tukey's *post hoc* tests for OR, OP, western blot, and dendritic spine data. Statistical significance was set at $p < 0.05$ for all statistical tests, and trends were determined by $p < 0.10$.

3. Results

3.1. DH infusion of E_2 enhances memory in E3FAD and E3/4FAD, but not E4FAD, mice

3.1.1. Object recognition—To determine whether *APOE* genotype influences the ability of E_2 to facilitate memory for a previously seen object or location, ovariectomized female E3FAD, E3/4FAD, and E4FAD mice were tested in the OR and OP tasks 24 h or 4 h, respectively, after receiving a post-training bilateral dorsal hippocampal infusion of vehicle (veh; E3FAD n = 21, E3/4FAD n = 12, E4FAD n = 21) or E_2 (E3FAD n = 16, E3/4FAD n = 11, E4FAD n = 14). In OR, E_2-treated E3FADs (Fig. 1A; $t_{(15)} = 3.406$, $p = 0.004$) and

E3/4FADs (Fig. 1A; $t_{(10)} = 2.745$, $p = 0.0201$) spent significantly more time than chance (15 s) with the novel object during testing, indicating intact memory for the identity of the training objects. By contrast, E_2-treated E4FADs, as well as all veh-treated groups, did not spend more time than chance with the novel object during testing, suggesting that two copies of the *APOE4* allele prevented E_2 from facilitating memory. A two-way ANOVA revealed a main effect of E_2 treatment (Fig. 1A; $F_{(1, 89)} = 9.614$, $p = 0.003$), as well as a significant genotype x treatment interaction ($F_{(2, 89)} = 4.821$, $p = 0.01$) for time spent with the novel object during testing. These effects were driven by the selective benefits of E_2 on OR memory in E3FAD and E3/4FAD females only. Tukey's *post hoc* comparisons support the notion that E_2-treated E3FAD and E3/4FADs drove the main effect of treatment, as there was a significant difference between vehicle- and E_2-treated E3/4FADs ($p < 0.031$), and a trend for a difference between vehicle-and E_2-treated E3FADs ($p = 0.07$).

Interestingly, time to accumulate 30 s of object exploration during testing was significantly influenced by genotype (Fig 1C; $F_{(2, 86)} = 11.93$, $p < 0.0001$), such that E4FADs took significantly less time to reach 30 s of object exploration relative to other groups. E4FADs treated with E_2 were particularly fast to complete object exploration, as demonstrated by *post hoc* comparisons to other E_2-treated groups (Fig 1C; $p = 0.002$ vs E_2-treated E3FADs, and $p = 0.0004$ vs E_2-treated E3/4FADs). Given the significant genotype effect during testing, we also examined time to accumulate 30 s of object exploration during training, but found no significant effects (Veh-treated E3FADs, M = 541.35, SEM = 56.359; E_2-treated E3FADs, M = 537.957, SEM = 48.977; Veh-treated E3/4FADs, M = 447.892, SEM = 65.291; E_2-treated E3/4FADs, M = 538.325, SEM = 70.724; Veh-treated E4FADs, M = 557.613, SEM = 71.566; E_2-treated E4FADs, M = 382.463, SEM= 57.742). This pattern of data suggests that E_2-treated E4FADs were not generally hyperactive, but rather just quick to explore objects during testing.

3.1.2. Object placement—The OP task yielded similar results to OR in that E_2 treatment selectively improved memory in E3FAD and E3/4FAD females. E_2-treated E3FAD (Fig. 1B; $t_{(12)} = 3.741$, $p = 0.003$) and E3/4FAD (Fig. 1B; $t_{(10)} = 5.644$, $p = 0.0002$) females spent significantly more time than chance with the moved object during testing, indicating intact memory for the identity of the training objects. As in OR, E_2-treated E4FAD and veh-treated mice of all genotypes did not spend more time than chance with the moved object during testing, again suggesting that *APOE4* homozygotes were unresponsive to the mnemonic benefits of E_2. As with OR, the main effect of treatment was significant (Fig. 1B; $F_{(1, 88)} = 8.183$, $p = 0.005$), as was the genotype x treatment interaction (Fig. 1B; $F_{(2, 88)} = 3.591$, $p = 0.032$), reflecting the selective benefit of E_2 for E3FAD and E3/4FAD females. However, unlike OR, the main effect of genotype was also significant (Fig. 1B; $F_{(2, 88)} = 10.72$, $p < 0.0001$), such that E3FAD and E3/4FADs exhibited better memory for object location than E4FADs. In support of these conclusions, Tukey's *post hoc* comparisons indicated that E_2-treated E4FADs spent significantly less time with the moved object than E_2-treated E3FADs (Fig. 1B; $p = 0.002$) and E3/4FADs (Fig. 1B; $p = 0.0009$).

Similar to OR, time to accumulate 30 s of object exploration during testing was significantly modulated by genotype (Fig 1D; $F_{(2, 89)} = 8.765$, $p = 0.0003$), such that E4FADs were faster to accumulate 30 s of object exploration relative to other groups. Exploration time in E_2-

treated E4FADs was significantly lower than that of E_2-treated E3FADs (Fig 1D; $p = 0.007$). We again examined time to accumulate 30 s of object exploration during training, but found no significant effects (Veh-treated E3FADs, M = 495.968, SEM = 59.455; E_2-treated E3FADs, M = 400.2, SEM = 56.820; Veh-treated E3/4FADs, M = 431.2, SEM = 77.794; E_2-treated E3/4FADs, M = 399.033, SEM = 34.655; Veh-treated E4FADs, M = 430.119, SEM = 67.464; E_2-treated E4FADs, M = 326.927, SEM = 36.685).

Combined, data from both OR and OP tasks suggest that E_2 supports memory consolidation in E3FAD, and E3/4FAD, but not E4FAD, females.

3.2.1. ERα is elevated in the DH of E4FAD mice—Because we previously found that *APOE4* increased ERα expression in the DH of gonadally-intact EFAD mice of both sexes (Taxier et al., 2022a), we examined effects of DH vehicle or E_2 infusion on levels of this protein and two other estrogen receptors, ERβ and G-protein-coupled estrogen receptor (GPER). Two-way ANOVA revealed a significant main effect of genotype, but not treatment, nor an interaction, for levels of ERα (Fig. 2A,B; $F_{(2,48)} = 6.956$, $p = 0.002$), driven by elevated ERα in E_2-treated E4FADs relative to veh-treated E3FADs ($p = 0.019$ veh-treated E3FADs vs E_2-treated E4FADs, $p = 0.052$ veh-treated E3FADs vs veh-treated E4FADs). There were no significant effects of treatment or genotype in DH protein expression of either ERβ or GPER (Fig. 2A,C,D).

3.2.2. ERK and JNK are not rapidly phosphorylated by E_2 in EFAD mice—In wild type C57BL/6 mice, the memory-enhancing effects of E_2 are facilitated by ERα and ERβ signaling via phosphorylation of extracellular signal-regulated kinase (ERK) (Boulware et al., 2013). Although activation of GPER also facilitates memory, it does so independently from ERK and from DH E_2 infusion via c-jun N-terminal kinase (JNK) activation (Kim et al., 2016). Because the cell-signaling events underlying E_2-facilitated memory are uncharacterized in EFADs, we examined whether E_2 facilitates memory via activation of ERK or JNK signaling in this model. Surprisingly, there were no significant effects of E_2 or genotype on ERK or JNK phosphorylation in the DH (Table 2).

3.3. DH and mPFC spine densities are affected by APOE genotype and E_2 treatment

We next examined apical and basal dendritic spine density on pyramidal neurons in hippocampal area CA1 and in the prelimbic/infralimbic area of the mPFC to assess whether *APOE* genotype influenced the ability of E_2 to increase dendritic spine density 2 h following DH infusion. Representative images of vehicle- and E_2-treated spines for each genotype are shown in Figs. 3A, 4A, and 5A, whereas representative images of mushroom, stubby, and thin spines are shown in Fig. 3B. There were no effects of genotype or E_2 treatment, nor an interaction, on mPFC apical spines (Table 1). Treatment and genotype effects on CA1 apical, and CA1 and mPFC basal spines, are detailed below.

3.3.1. CA1—*APOE4* homozygosity blocked E_2's effects on CA1 total apical spine density, as suggested by the fact that E_2 significantly increased CA1 total apical spine density in E3FADs and E3/4FADs, but not E4FADs. Significant main effects of genotype (Fig. 3C; $F_{(2,29)} = 6.746$, $p = 0.0039$) and E_2 treatment (Fig. 3C; $F_{(1,29)} = 30.25$, $p < 0.0001$)

were observed, as well as a genotype x treatment interaction (Fig. 3C; $F_{(2,29)} = 4.536$, $p = 0.0193$). Tukey posthoc tests revealed that total apical spine density was significantly higher in E3FAD ($p = 0.0032$) and E3/4FAD ($p = 0.0033$) mice treated with E_2 than with vehicle, whereas there were no effects of E_2 found in E4FAD mice ($p = 0.932$). Moreover, apical spine density was significantly lower in E_2-treated E4FAD mice than in E_2-treated E3FAD ($p = 0.0451$) and E3/4FAD mice ($p = 0.002$). Further analysis by spine subtype revealed no significant effects of genotype or treatment, nor interactions, on CA1 apical thin or mushroom spines (Table 1). However, the main effects of genotype (Fig. 3D; $F_{(2,31)} = 4.261$, $p = 0.0002$) and treatment (Fig. 3D; $F_{(1,31)} = 9.712$, $p = 0.012$) were significant for CA1 apical stubby spines. Here, the only significant between-group differences were between the E3FAD and E4FAD vehicle groups, such that veh-treated E3FAD mice had more stubby apical spines than veh-treated E4FAD mice ($p = 0.006$).

CA1 basal spine density was influenced by genotype only. The main effect of genotype was significant (Fig. 4B; $F_{(2,31)} = 7.774$, $p = 0.002$), but the treatment effect and interaction were not. This effect was driven largely by elevated total spine density in E_2-treated E3FAD mice, who had significantly higher basal spine density than E_2-treated E4FADs (Fig. 4B; $p = 0.002$). Further analysis by spine subtype revealed that the main effect of genotype on total CA1 basal spine density was driven by significant effects of genotype on CA1 basal stubby spines (Fig. 4C; $F_{(2,31)} = 4.49$, $p = 0.019$) and thin spines (Fig. 4D; $F_{(2,31)} = 7.887$, $p = 0.002$). E_2-treated E3FADs exhibited elevated thin basal spines relative to E_2-treated E4FADs (Fig. 4D; $p = 0.013$). The genotype x treatment interaction was significant for CA1 basal mushroom spines (Fig. 4E; $F_{(2, 30)} = 5.151$, $p = 0.012$), such that mushroom spine density was elevated in E_2-treated E3FADs relative to E_2-treated E4FADs (Fig. 4E; $p = 0.012$).

3.3.2. mPFC—Neither genotype nor E_2 treatment affected mPFC apical spine density in terms of total spines or spine subtypes (Table 1). However, there were modest effects of genotype on mPFC basal spines, such that E4FADs again had the lowest spine density. For total basal spines, a two-way ANOVA revealed a trend towards a significant main effect of genotype (Fig. 5B; $F_{(2, 30)} = 3.178$, $p = 0.055$), which appeared to be driven by a significant reduction among E4FAD mice in thin spines (Fig. 5C; $F_{(2, 30)} = 5.212$, $p = 0.011$). Although no *post hoc* comparisons were significant, E3FADs generally had elevated mPFC basal thin spines relative to EFADs of other genotypes, regardless of treatment. There were no significant genotype, treatment, nor genotype x treatment effects on levels of mPFC basal stubby or mushroom spines (Table 1).

4. Discussion

Because *APOE4* status and female sex are unmodifiable risk factors that act synergistically to increase AD risk (Altmann et al., 2014), evaluating interventional approaches that might benefit female *APOE4* carriers is a crucial step towards providing individualized treatment to AD patients. Here, we investigated whether *APOE* genotype interacts with the ability of E_2 to promote memory consolidation, modulate estrogen receptor expression, and increase CA1 or mPFC dendritic spine density in an EFAD mouse model of AD designed to model *APOE*-associated disease risk against a background of AD-like pathology. We hypothesized

that E_2 would have the most beneficial effects on memory consolidation and spine density in ovariectomized E3FAD mice, and that E_2 may exert lesser effects in E3/4FAD mice due to the presence of one *APOE4* allele. Based on data from women showing no protective effect of estrogen therapy on cognitive decline in *APOE4* carriers (Yaffe et al., 2000), we expected that E_2 would have little to no influence on memory or spine density in E4FAD females and intermediate effects in E3/4FAD mice due to the presence of one *APOE4* allele. We found that DH infusion of E_2 facilitated object memory consolidation in both E3FAD and E3/4FAD mice, but not E4FAD mice, indicating that two copies of the *APOE4* allele blocks the memory-enhancing effects of E_2. The effects of E_2 in E3FAD and E3/4FAD females were associated with an increase in total CA1 apical spine density. Independent of E_2 treatment, *APOE4* genotype alone reduced CA1 and mPFC spine density and increased levels of ERα in the DH.

The beneficial effects of acute DH E_2 infusion on object recognition and spatial memory consolidation in 6-month-old ovariectomized E3FAD and E4FAD mice are consistent with an extensive literature from our lab and others showing similar benefits of DH E_2 infusion in 2–3-month-old wild-type ovariectomized C57BL/6 mice (Fernandez et al., 2008; Fortress et al., 2013; Gross et al., 2021; Packard and Teather, 1997; Taxier et al., 2019). A primary difference is that the delays between training and testing used here (24 h for OR and 4 h for OP) were shorter than those at which E_2 benefits wild-types (48 h for OR and 24 h for OP), but this difference is to be expected given the significant AD-like pathology present in the brains of EFAD mice (Tai et al., 2017; Youmans et al., 2012). Although others have shown a potential benefit of chronic E_2 treatment for decreasing amyloid accumulation in ovariectomized EFAD mice (Kunzler et al., 2014) and in ovariectomized 3xTg mice (Carroll et al., 2007), our data are the first to demonstrate that a single acute dose of E_2 can facilitate memory consolidation in E3FAD and E3/4FAD, but not E4FAD mice. Previous work demonstrated that chronic E_2 treatment initiated in 3-month-old EFADs can significantly reduce amyloid burden (Kunzler et al., 2014). We find it particularly striking that E3FAD and E3/4FAD mice remain responsive to a single acute dose of E_2 until at least 6 months of age, at which point there is already substantial AD-like pathology evident (Tai et al., 2017). Although recent clinical studies suggest that estrogen therapy is not efficacious for women with AD, these findings are clouded by differences in treatment regimen, age of experimental subjects and menopausal status, and lack of stratification by *APOE* status in some instances. Thus, whether *APOE3*+ women with AD who express 0 or 1 copies of *APOE4* can benefit cognitively from E_2 treatment after substantial disease progression remains a question for future study.

Although there was a significant interaction between genotype and treatment in both the OR and OP tasks, the pro-cognitive effects of E_2 were more apparent for OP, suggesting that spatial memory may be more sensitive to E_2 in ovariectomized E3FAD and E3/4FAD mice than recognition memory. Alternatively, the longer 24-hour delay between training and testing for OR may have been more demanding on memory function than the shorter 4-hour delay for OP. Interestingly, E4FAD mice took less time than other genotypes to accumulate 30 seconds of object exploration during testing, but not during training, which is an unusual finding in our extensive experience using these tasks to examine estrogenic regulation of memory. One possible explanation is that E4FAD mice noticed a change during testing that

caused an increase in exploratory behavior, but without the ability to discriminate between old and new objects and locations, this increase did not result in a preference for novelty. Our previous work with gonadally-intact female EFAD mice indicated that during object memory habituation trials, E4FAD females explored the outer third of the open field box more than E3FAD females (Taxier et al., 2022b). Our objects are located in this peripheral zone, and so a possible training-induced increase in exploration in this zone could have caused this group to contact and explore the objects faster than other groups that were exploring the entirety of the testing arena. Future studies will need to further address this possibility.

Importantly, our results also suggest that individuals carrying a single copy of the *APOE4* allele may still benefit from the cognitive benefits of E_2 treatment, whereas individuals with two copies would not. This finding is somewhat consistent with data from human patients, from which the conclusion is sometimes reached that estrogens are ineffectual or in some instances harmful for cognition in *APOE4* carriers (Burkhardt et al., 2004; Kang and Grodstein, 2012). Others have reported neuroprotective effects and cognitive benefits of estrogens in human *APOE4* carriers, especially when treatment is initiated within a critical window (Jacobs et al., 2013; Mahoney et al., 2020). Because individuals carrying two *APOE4* alleles are relatively rare in human populations (Heffernan et al., 2016), therefore making sufficiently-powered sample size a challenge, studies with human patients do not often differentiate between women carriers of one or two *APOE4* alleles (but see Mahoney et al., 2020). Data from the present study suggests that this distinction is important because heterozygous E3/4FAD female mice responded to E_2, whereas homozygous E4FADs did not. More work remains to determine what factors mediate the neuroprotective properties of E_2 in carriers of one or both copies of an *APOE4* allele.

Surprisingly, the memory-enhancing effects of E_2 in the present study occurred in the absence of rapid p42 ERK phosphorylation in the DH, which has been documented many times in young adult wild type C57BL/6 mice and is necessary in these mice for E_2 to enhance memory consolidation in the object recognition and object placement tasks (e.g., Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2013). Therefore, data from the current study suggest that the benefits of E_2 in ovariectomized E3FAD and E3/4FAD mice occur either via a different time course of ERK activation or an as-of-yet uncharacterized estrogen-sensitive signaling event. The absence of rapid JNK phosphorylation in the present study also suggests GPER-independent signaling, given that the effects of GPER activation on object recognition and object placement memory consolidation in young ovariectomized wild type mice are dependent upon activation of this kinase (Kim et al., 2016, 2019). It is possible that pathological changes in the DH of these mice (e.g., Youmans et al., 2012) altered the timing of kinase activation relative to wild types, so collecting tissue from E_2-infused EFADs at extended time points would allow for a more complete picture of E_2-related cell signaling across genotypes.

Our previous study of gonadally-intact male and female E3FAD and E4FAD mice revealed that E4FADs of both sexes had significantly higher levels of ERα in the DH (Taxier et al., 2022a). The present study is consistent with this finding and extends it to ovariectomized E4FAD mice. Our data thus far suggest that the increase in ERα among E4FAD mice is

associated with impaired memory consolidation (Taxier et al., 2022a) and an inability of E_2 to enhance memory consolidation (present study). Consistent with these data, others have shown that ERα is elevated in the hippocampus of AD patients, particularly *APOE4* carriers (Ishunina and Swaab, 2003). It is unclear why this is so. It could be that ERα is increased in response to changes in levels of other ERs. However, levels of ERβ and GPER were unaffected by genotype or treatment, so this is unlikely. The increase in ERα could come in response to reduced levels of E_2 synthesized within the hippocampus. Levels of E_2 and mRNA levels of the E_2-synthesizing enzyme aromatase were lower in frontal cortices and cerebella of postmortem brains from female AD patients than in non-AD controls (Yue et al., 2005), supporting the possibility that ERα levels may increase in an attempt to compensate for lower levels of brain E_2. However, it is unknown if or how levels of E_2 or aromatase are altered by genotype in EFAD mice. Thus, the relationship between *APOE4* homozygosity and ERα levels warrants future study, especially in light of previous findings from rat hippocampus that *APOE* mRNA and APOE protein expression are upregulated 24 h following ERα activation, either with E_2 or an ERα agonist (Wang et al., 2006). Future work should examine whether hippocampal *APOE* expression is altered following E_2 treatment in E3FAD, E3/4FAD, or E4FADs. With respect to the lack of E_2 effects on ER levels in the present study, it should be noted that 5 minutes is a very rapid timepoint at which to see effects on receptor expression of a single E_2 treatment, so future studies should examine longer time points and/or longer durations of E_2 treatment. Better understanding the extent to which levels of the three ERs are altered by genotype and in response to E_2 treatment across genotypes could provide valuable information as to whether selective estrogen receptor modulators could be potentially useful therapeutics for preventing or treating cognitive decline in *APOE4*+ women.

The effects of E_2 on CA1 apical dendritic spine density were remarkably consistent with its beneficial effects on memory. In line with its memory-enhancing effects in ovariectomized E3FAD and E3/4FAD females, E_2 increased CA1 total apical spine density in E3FAD and E3/4FAD, but not E4FAD, females two hours after infusion. This effect is consistent with previous findings from our lab showing that CA1 total apical spine density is increased two hours after E_2 infusion in wild-type ovariectomized mice (Tuscher et al., 2016). Here, we also showed that E_2 modestly increased CA1 apical stubby spine density in EFADs, although levels of apical stubby spines were similar in E3FADs regardless of treatment. Although thin spines are typically considered more likely than stubby spines to transition to other spine types, previous work in another AD model suggests that stubby spines can similarly take on this dynamic role in pathological conditions (Spires-Jones et al., 2007). Therefore, an increase in apical stubby spines in CA1 may reflect ongoing spine remodeling or spinogenesis stimulated by E_2. Interestingly, E_2 also increases CA1 and mPFC basal spines two hours post intrahippocampal E_2-infusion in wild-type ovariectomized mice (Tuscher et al., 2016). However, we did not see a corresponding increase in CA1 and mPFC basal spines following E_2 infusion in E3FADs or E3/4FADs, which may be reflective of reduced interactions or connectivity between mPFC and CA1 in E_2-treated EFADs relative to wild-type mice. Nevertheless, our analyses suggest that E_2 may exert its memory-enhancing effects in E3FAD and E3/4FAD mice via increased CA1 apical spine density.

E_2 also had a striking effect on CA1 basal mushroom spines, for which the genotype x treatment interaction was significant. This finding, in which E_2-treated ovariectomized E3FADs had the highest number of CA1 basal mushroom spines, whereas E_2-treated E4FADs had the lowest, represents a key measure by which E_2 benefits *APOE3* carriers, while harming *APOE4* carriers. Because mushroom spines are thought to be mature spines (Bourne and Harris, 2007; Hayashi and Majewska, 2005), the beneficial effects of E_2 on memory in E3FADs, and its inefficacy in E4FADs, may be linked to the respective E_2-induced increase of CA1 mushroom spines in E3FADs, and E_2-induced decrease of this same spine subtype in E4FADs. That is, in addition to effects on CA1 apical spines, E_2 may facilitate memory in E3FADs by increasing the density of mature spines on CA1 basal dendrites and impair memory in E4FADs by reducing basal mature spine density. However, this possibility is limited by the fact that the same effect of E_2 on CA1 basal mushroom spines was not present in E3/4FADs at the single time point we examined. Recent work suggests that dendritic spine loss does not occur at random in AD patients; rather, spines are lost in clusters depending on the presence or absence of tau pathology in examined cells (Mijalkov et al., 2021). Therefore, because cells and dendritic segments in the present study were semi-randomly chosen for analyses based on stain penetrance, our analyses are limited by the fact that we do not know for certain whether spines were counted from neurons in which AD-like pathology was present. The use of advanced methods such as immuno-EM in tissue collected from EFADs could provide ultrastructural information about the presence or absence of pathological hallmarks of AD in specific cellular compartments (Takahashi et al., 2010).

Genotype modulated spine density in a manner largely consistent with our hypotheses, in that genotype effects on CA1 apical, CA1 basal, and mPFC basal spines were seemingly driven by increased dendritic spine density in E3FADs, or decreased dendritic spine density in E4FADs, relative to other groups, which may have contributed to their impaired memory consolidation. The main effect of genotype on basilar thin spine density in CA1 is of particular interest because *post hoc* comparisons indicated that E_2-treated E3FADs had the highest density of this subtype. Our data are consistent with previous work showing that treatment with E_2 facilitated cognitive function in aged rhesus monkeys, an effect dependent upon increased thin spine density (Hao et al., 2006). Furthermore, our data fit with the notion that thin spines are thought to be “learning spines,” or transient protrusions that often evolve into more mature, mushroom “memory” spines (Bourne and Harris, 2007). CA1 basal and proximal apical dendrites receive projections from CA3 to support memory processes (Li et al., 1994; Spruston, 2008; Whitlock et al., 2006), and E_2 treatment evokes an increase in synaptic transmission at CA3-CA1 synapses (Kumar et al., 2015; Smejkalova and Woolley, 2010; Smith et al., 2009). Therefore, higher density of CA1 total apical spines in E_2-treated E3FADs and E3/4FADs, as well as higher density of CA1 basilar thin spines in E_2-treated E3FADs, could be linked to improved memory processes. The lack of genotype or treatment effects on mPFC apical spine density (Table 1), and modest genotype effects on mPFC basal spine density indicate that the memory-enhancing effects of E_2 in ovariectomized E3FADs and E3/4FADs are not mediated through changes in mPFC spine density. However, the genotype effects on mPFC total and thin basal spines suggests that E3FAD females have somewhat higher basal spine density relative to E4FAD females,

which is consistent with our previous findings in gonadally-intact E3FAD and E4FAD mice (Taxier et al., 2022a).

In sum, intrahippocampal E_2 infusion facilitated memory consolidation and increased CA1 apical spine density in ovariectomized E3FAD and E3/4FAD mice, suggesting that mice with AD-like pathology bearing zero or one copy of *APOE4* remain responsive to acute E_2 treatment. Notably, two copies of *APOE4* had a deleterious effect on the ability of E_2 to enhance memory and increase dendritic spine density, and also increased levels of ERα in a manner possibly linked to impaired memory consolidation. In addition, the fact that E_2 increased CA1 basal mushroom spine density in E3FADs and reduced these spines in E4FADs may account for the respective ability and inability of E_2 to enhance memory in E3FADs and E3/4FADs versus E4FADs. These data support the notion that E_2 treatment can potentially benefit individuals who carry zero or one copy of the *APOE4* allele, but not those with two copies. Future experiments should examine the kinetics of both protein expression and dendritic spine density following E_2 treatment at multiple time points to fully elucidate the molecular correlates of the mnemonic benefits of E_2 in EFAD mice. Much more work remains to fully characterize the interactive effects between E_2 and *APOE4* genotype and to determine how *APOE4* impedes the actions of E_2. This information may provide new important new insights about the impact of E_2 loss on AD risk in women and better define subpopulations of women who could benefit cognitively from estrogen therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5xFAD	5 familial AD mutations
AD	Alzheimer's disease
ANOVA	analysis of variance
DH	dorsal hippocampus
E_2	17β-estradiol
EFAD	$APOE^{+/+}$/5xFAD$^{+/-}$
E3FAD	$APOE^{3/3}$/5xFAD$^{+/-}$
E3/4FAD	$APOE^{3/4}$/5xFAD$^{+/-}$

E4FAD	*APOE*$^{4/4}$/5xFAD$^{+/-}$
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
GPER	G-protein-coupled estrogen receptor
mPFC	medial prefrontal cortex
OP	object placement
OR	object recognition
Tg	transgenic
s	second
min	minute
h	hour

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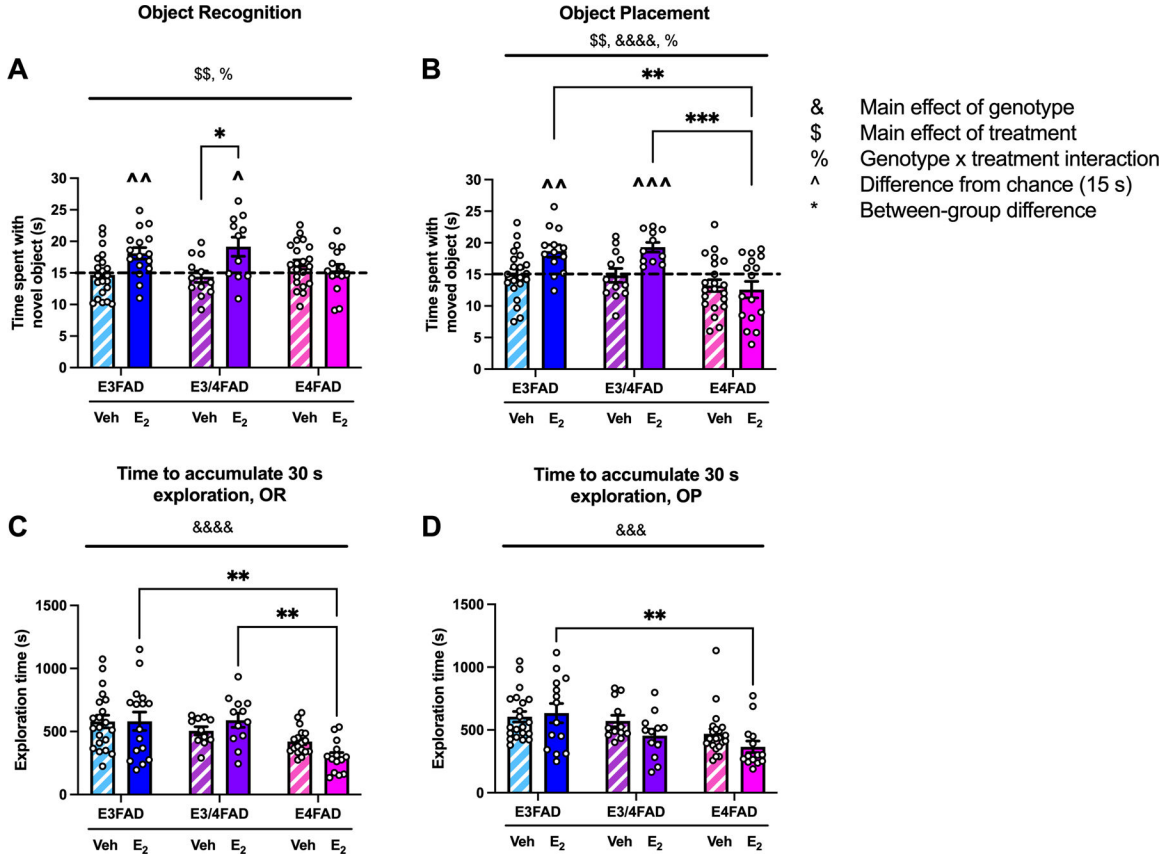

Figure 1. E_2 facilitates object recognition and spatial memory formation in E3FAD and E3/4FAD, but not E4FAD, females.

(A,B) E3FAD and E3/4FAD females treated with E_2 spent significantly more time than chance (dashed line at 15 sec; ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$) with the novel (A) and moved (B) objects during testing. In contrast, E_2-treated E4FAD females did not, nor did any groups treated with vehicle (veh). The genotype-dependent effect of E_2 on memory was also reflected in between-subjects analyses by a main effect of treatment ($^{\$\,\$}p < 0.01$) and treatment x genotype interaction ($^{\%}p < 0.05$). In OP, the main effect of genotype ($^{\&\&\&\&}p < 0.0001$) indicated that E3FADs and E3/4FADs spent more time with the moved object than E4FADs. Tukey's *post hoc* comparisons revealed significant differences between veh- and E_2-treated E3/4FADs in OR (A; $*p < 0.05$), and that E_2-treated E3FADs ($**p < 0.01$) and E3/4FADs ($***p < 0.001$) spent more time with the moved object in OP than E_2-treated E4FADs. (C,D) The main effect of genotype was significant for both OR (C; $^{\&\&\&\&}p < 0.0001$) and OP (D; $^{\&\&\&}p < 0.001$). E4FADs took less time to accumulate 30 sec of exploration in both tasks. Tukey's *post hoc* comparisons indicated that E_2-treated E4FADs were faster than E_2-treated E3/4FADs and E3FADs in OR (C; $**p < 0.01$), and than E_2-treated E3FADs in OP (D; $**p < 0.01$). Bars represent mean ± standard error of the mean (SEM).

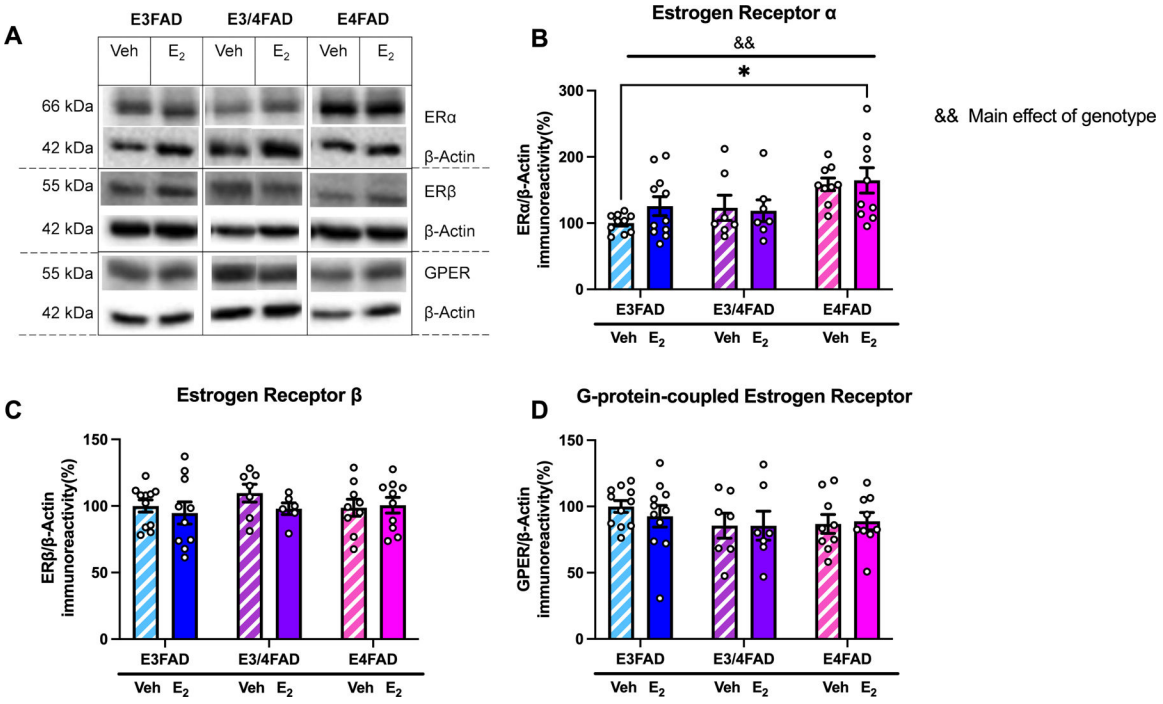

Figure 2. ERα levels in the DH were increased in E4FAD females.

(A) Representative Western blot images. (B) ERα was affected by *APOE* genotype ($^{\&\&}p < 0.01$ = main effect of genotype) such that levels were highest in E4FAD females. ERα levels were significantly higher in E_2-treated E4FAD mice than in veh-treated E3FAD mice ($^{*}p < 0.05$). (C,D) Levels of ERβ (C) and GPER (D) were unaffected by treatment or genotype.

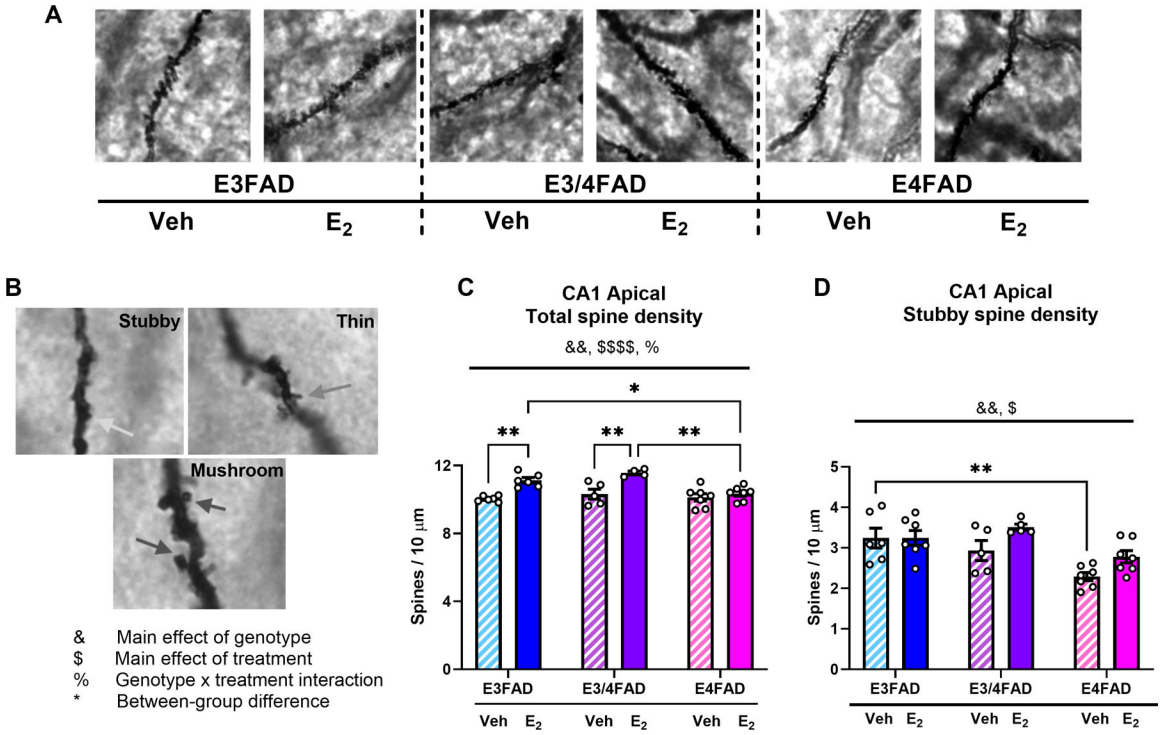

Figure 3. E_2 increases CA1 apical spine density in E3FAD and E3/4FAD, but not E4FAD, females.

(A) Representative images of CA1 apical dendritic spine segments at 100X magnification. (B) Representative images of stubby (top left), thin (top right), and mushroom (bottom) spines. Arrows indicate individual examples of each spine class. (C) Total CA1 apical dendritic spine density was increased by DH E_2 infusion ($^{\$\$\$\$}p < 0.01$ = main effect of treatment) and reduced by two copies of *APOE4* ($^{\&\&}p < 0.05$ = main effect of genotype; $^{\%}p < 0.05$ = interaction). In both E3FAD and E3/4FAD females, E_2 treatment increased total apical spine density relative to veh ($^{**}p < 0.01$), but no benefit of E_2 was observed in E4FAD mice. Moreover, apical spine density was significantly lower in E_2-treated E4FAD mice than in E_2-treated E3FAD ($^{*}p < 0.05$) and E3/4FAD ($^{**}p < 0.01$) mice. (C) CA1 apical stubby spines was modestly increased by E_2 ($^{\$}p < 0.05$ = main effect of treatment) and reduced by two copies of *APOE4* ($^{\&\&\&}p < 0.001$ = main effect of genotype). No differences were observed between veh and E_2 within any genotype, but veh-treated E3FADs had significantly more stubby spines than veh-treated E4FADs ($^{**}p < 0.01$).

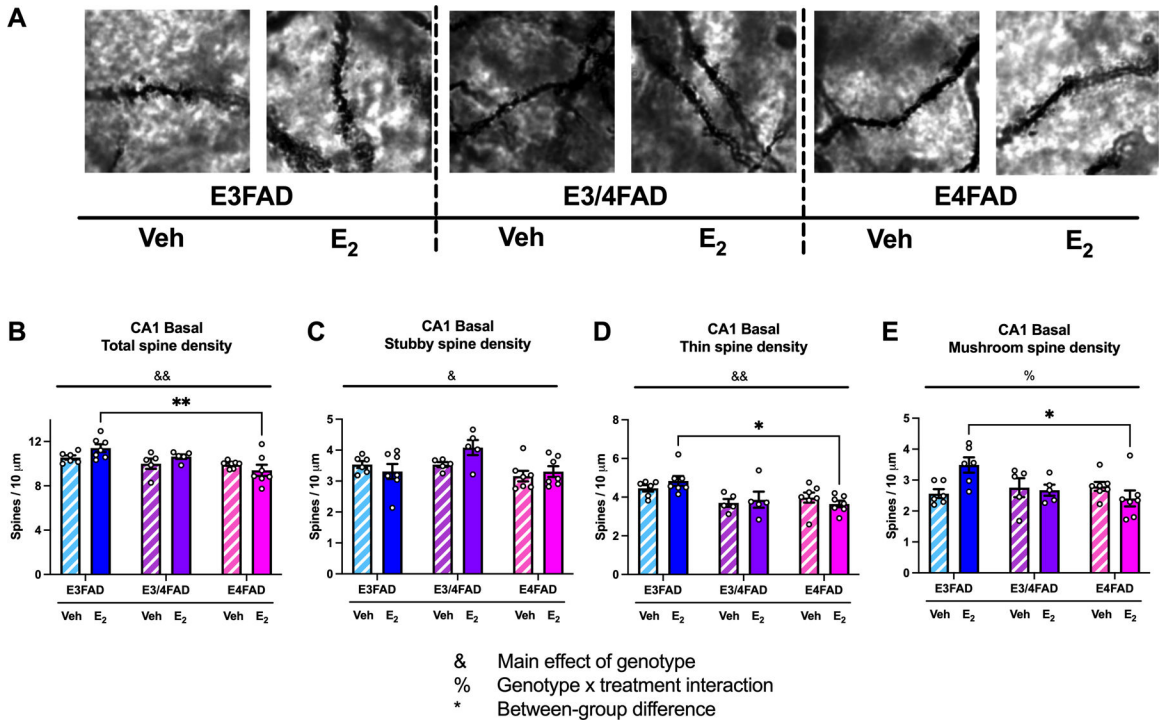

Figure 4. CA1 basal spine density was reduced by *APOE4*.

(A) Representative images of CA1 basal dendritic spine segments at 100X magnification. (B-E) Total CA1 basal spine density (B), stubby spine density (C), and thin spine density (D) was decreased in E4FADs, regardless of treatment ($^{\&}p < 0.05$, $^{\&\&}p < 0.01$ = main effect of genotype). The treatment x genotype interaction was significant for mushroom spines (E; $^{\%}p < 0.05$). E_2-treated E3FAD females had more total spines, thin spines, and mushroom spines than E_2-treated E4FAD females. ($^{*}p < 0.05$, $^{**}p < 0.01$).

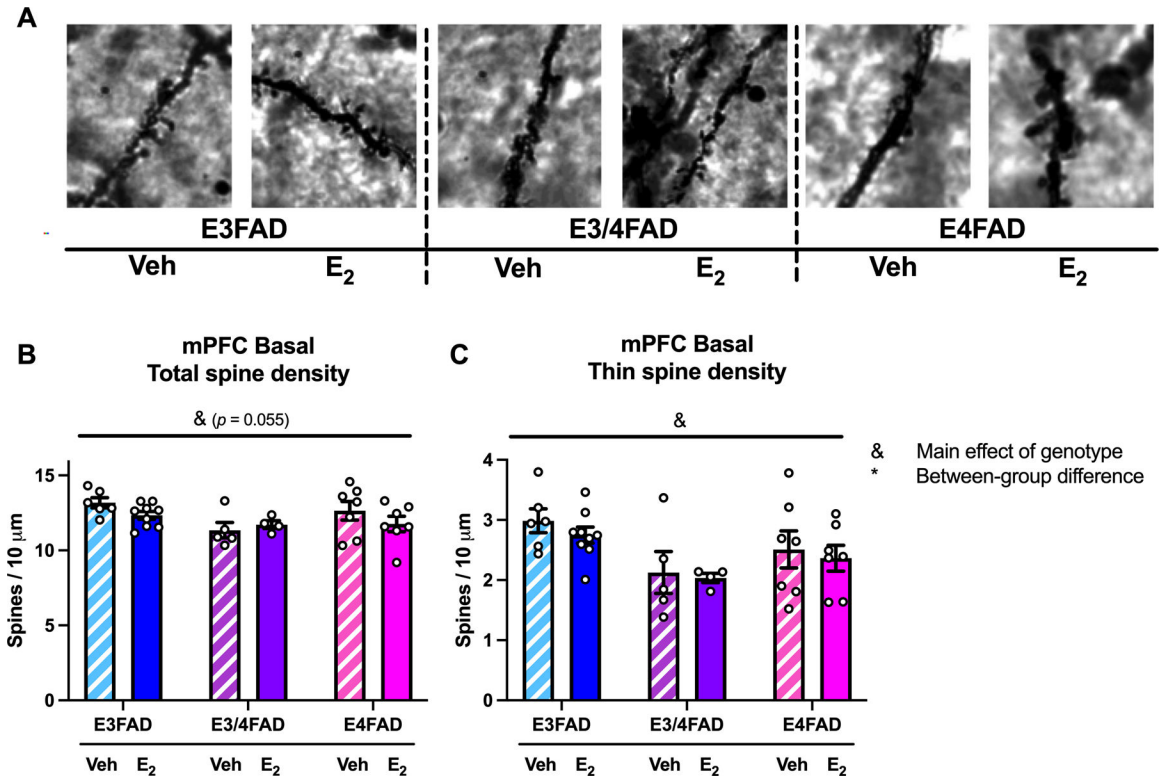

Figure 5. mPFC basal spine density was reduced by *APOE4*.

(A) Representative images of mPFC basal dendritic spine segments at 100X magnification. (A) *APOE4* genotype reduced mPFC basal spines, as suggested by a modest main effect of genotype for total spines (B; $^{\&}p = 0.055$) and a significant main effect in thin spines (C; $^{\&}p < 0.05$). No between-group differences were observed.

Table 1.

Levels of phosphorylated ERK and JNK were unaffected by E_2 treatment or genotype

Protein*	E3FAD		E3/4FAD		E4FAD	
	Veh	E_2	Veh	E_2	Veh	E_2
p42 ERK	100.0 ± 10.56	93.21 ± 10.05	109.39 ± 20.84	112.56 ± 16.44	109.45 ± 15.76	110.17 ± 14.46
p44 ERK	100.0 ± 9.32	119.51 ± 8.41	97.37 ± 12.20	124.85 ± 9.37	133.63 ± 18.73	111.44 ± 9.96
p46 JNK	100.0 ± 2.05	89.52 ± 3.88	93.15 ± 6.28	94.97 ± 4.50	102.83 ± 5.25	94.02 ± 4.60
p54 JNK	100.0 ± 5.86	89.70 ± 7.51	96.39 ± 8.67	90.60 ± 7.57	97.68 ± 7.94	8.35 ± 8.05

*All proteins were normalized to the vehicle-treated E3FAD group, whose values were set to 100.

Values represent mean % immunoreactivity (± SEM) for the target phosphorylated protein divided by unphosphorylated total protein.

Table 2.

CA1 and mPFC spine density measures unaffected by E_2 treatment or genotype

Brain region	Spine type	E3FAD		E3/4FAD		E4FAD	
		Veh	E_2	Veh	E_2	Veh	E_2
CA1	Apical thin	3.69 ± 0.18	4.51 ± 0.29	4.06 ± 0.28	4.02 ± 0.11	4.26 ± 0.27	4.32 ± 0.21
	Apical mushroom	3.05 ± 0.11	3.06 ± 0.25	3.30 ± 0.23	3.71 ± 0.30	3.52 ± 0.28	3.20 ± 0.11
mPFC	Apical total	11.6 ± 0.88	10.35 ± 0.46	11.43 ± 0.95	10.03 ± 0.72	10.19 ± 0.85	9.84 ± 0.48
	Apical thin	2.74 ± 0.24	2.27 ± 0.06	2.41 ± 0.26	2.24 ± 0.10	2.75 ± 0.28	2.51 ± 0.14
	Apical stubby	5.32 ± 0.48	4.77 ± 0.32	5.42 ± 0.43	4.95 ± 0.59	4.59 ± 0.55	4.46 ± 0.38
	Basal stubby	7.36 ± 0.46	6.63 ± 0.28	6.80 ± 0.26	6.95 ± 0.57	7.82 ± 0.38	6.79 ± 0.24
	Basal mushroom	2.84 ± 0.20	2.96 ± 0.23	2.42 ± 0.32	2.73 ± 0.50	2.32 ± 0.27	2.61 ± 0.29

Values represent mean spines/10 μM ± SEM