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Interactions Among Sex, Apolipoprotein E Genotype, and 17-Beta Estradiol in a Mouse Model of Alzheimer's Disease

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INTERACTIONS AMONG SEX, APOLIPOPROTEIN E GENOTYPE, AND 17-β ESTRADIOL IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

by

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ABSTRACT

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Under the Supervision of Professor Karyn Frick

Alzheimer's disease (AD) is the most common form of dementia, yet its cause remains a mystery and no truly effective treatments exist. The high incidence of AD, coupled with its devastating health and economic impacts, highlight the urgent need for continued research into the etiology of this disease (Ernst & Hay, 1994; Rice et al., 1993). Although existing and efficacious treatments for AD are lacking, several risk factors for AD have been identified. One such factor is apolipoprotein E genotype, which is the greatest genetic risk factor for AD (Ertekin-Taner, 2007; van der Flier et al., 2011). Another factor is female sex; women comprise almost two-thirds of AD cases (Alzheimer's Association, 2021), illustrating a need to understand whether there are sex and gender-specific risk factors for AD. Importantly, female sex acts synergistically with *APOE4* genotype to increase disease risk (Altmann et al., 2014; Bretsky et al., 1999; Farrer et al., 1997). Considerable attention has been given to sex steroid hormones such as the potent estrogen 17-β estradiol (E_2) as potential mediators of AD risk in females, especially given memory decline that coincides with the onset of the menopausal transition (Jacobs et al., 2016; Paganini-Hill & Henderson, 1996). However, interactions among sex, E_2, and *APOE* genotype remain poorly characterized, both in

human patients and in animal models of disease. The overall aim of this dissertation was to determine whether sex and estrogens influence memory consolidation and cognition, cell signaling, and dendritic morphology in a mouse model of AD designed to recapitulate *APOE*-associated disease risk. To this end, we used male and female transgenic mice that express 5 familial AD mutations (5xFAD) and human *APOE3* (E3FAD) or *APOE4* (E4FAD). We first tested 6 month-old male and female E3FAD and E4FAD mice in a behavioral battery of tasks including object recognition (OR), object placement (OP), open field (OF), and the Morris water maze, in order to assess whether female sex and *APOE4* genotype act independently or together to increase anxiety-like behavior and interfere with memory consolidation. We then examined the brains of these mice to ask whether sex or *APOE4* genotype, independently or concurrently, modulated changes in protein expression or dendritic spine density. Next, we asked whether gonadal status modulated anxiety-like behavior in female EFADs. Finally, we asked whether post-training intrahippocampal administration of E_2 was of mnemonic benefit to female E3FADs, E3/4FADs, and E4FADs for object memory consolidation, and whether this treatment resulted in changes in protein expression or dendritic spine density in the brains of these mice. Collectively, our results suggest surprisingly few effects driven by sex alone, or interactions between sex and *APOE4* genotype. However, *APOE4* genotype was memory-impairing, associated with decreased spine density, and altered protein expression relative to *APOE3* genotype. Furthermore, whereas E_2 facilitated memory consolidation in ovariectomized E3FAD and E3/4FAD females, E4FAD females were resistant to the memory-enhancing effects of E_2, including E_2 -induced changes in dendritic spine density. Combined, these data add to a

growing literature implicating *APOE4* as a harmful risk factor for AD, and suggest that E_2, and its downstream effectors, may be useful therapeutic targets for individuals not homozygous for *APOE4*.

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CHAPTER ONE: Introduction and background

Alzheimer's disease pathology and symptomology

AD is the leading cause of dementia worldwide (Alzheimer's Association, 2021). Patients with AD experience a broad array of symptoms that interfere with daily life, of which the most prominent is memory dysfunction. Current drugs available to treat AD, such as acetylcholinesterase inhibitors (e.g. donepezil or galantamine) and NMDA receptor antagonists (e.g. memantine) provide some symptom management, but do not reverse the course of disease, which is always fatal (Briggs et al., 2016). The newest approved drug for AD, aducanumab, is highly controversial and its efficacy for the cognitive symptomology of disease remains questionable (Phizackerley, 2021; Selkoe, 2019). Due to few efficacious treatment options that often do not substantially modulate cognition, the behavioral features of AD continue to contribute to poor quality of life for AD patients. Thus, understanding the mechanisms underlying memory dysfunction in AD is crucial for the development of better treatment strategies.

Additional behavioral manifestations of AD include increased affective and emotional dysregulation, which can also severely impair daily functioning (Ferretti et al., 2001; Porter et al., 2003). Although memory dysfunction is the predominant behavioral hallmark of AD, anxiety is a frequent comorbidity (Ferretti et al., 2001; Porter et al., 2003). Indeed, although the primary thrust of much AD research has been to uncover underpinnings of memory dysfunction, less is known about factors contributing to anxiety in AD patients. Given the prevalence of anxiety in AD patients (Ferretti et al., 2001), efforts should be made to include this symptom in disease treatment. Although anxiety may

result from confusion and distress caused by other cognitive dysfunction, its prevalence among AD patients underscores that this symptom should not be overlooked.

Apart from cognitive symptomology, AD is characterized by two neuropathological hallmarks: amyloid plaques and neurofibrillary tangles. These two disease hallmarks were first described by Alois Alzheimer in 1906 at the 37th Meeting of South-West German Psychiatrists. During this meeting, Alzheimer reported on a case study of a patient named Auguste D., whose health he had been monitoring since her admission five years prior to the Frankfurt Psychiatric Hospital (Cipriani et al., 2011; Stelzmann et al., 1995). Alzheimer performed an autopsy on the brain tissue of Auguste D. following her death. He described "military foci…which represented the sites of deposition of a peculiar substance," which were later identified as amyloid plaques, as well as fibrils surrounding neuronal cells, which we now know to be comprised of hyperphosphorylated tau (Cipriani et al., 2011; Stelzmann et al., 1995).

In the many years since this initial characterization of the AD brain, researchers have dedicated considerable effort towards understanding whether amyloid deposition and neurofibrillary tangles are a cause or an effect of AD. Although both of these neuropathological hallmarks often emerge before cognitive decline is apparent (Price & Morris, 1999), we lack an understanding of what precipitates amyloid and tau pathology, as well as the consequences arising from the presence of this pathology in the brain. Some researchers sit squarely in the amyloid hypothesis camp, some claim that tau hyperphosphorylation is the culprit of disease pathogenesis, and still others posit that amyloid and tau work synergistically to promote disease progression. Researchers arguing that amyloid and tau work together to contribute to disease sometimes use a

bullet/trigger analogy; that is, amyloid accumulation, which occurs earlier on, is the trigger, whereas tau, which forms neurofibrillary tangles later than amyloid plaque formation, is the bullet (Bloom, 2014). However, the distinct role that each of these pathological hallmarks plays in disease progression, disease onset, or disease-associated cognitive symptomology remains elusive. The presence of these hallmarks in non-demented individuals (Fagan et al., 2009; Morris et al., 2010) also indicates that AD is not necessarily the cause of, or caused by, these markers. Furthermore, numerous disease-modifying therapies aimed at reducing amyloid burden or tau hyperphosphorylation have failed clinical trials (Gauthier et al., 2016), necessitating identification of additional biomarkers, risk factors, and treatment targets.

The hippocampus and prefrontal cortex in AD

Although AD results in brain-wide dysfunction, learning and memory-related brain regions such as the hippocampus and the prefrontal cortex (PFC) are often implicated as major players in AD. The hippocampus is affected early on in disease progression, identifying this region as a key target for early detection and treatment of disease (Braak & Braak, 1991). Several landmark postmortem analyses of brains of AD patients revealed that hippocampal projection neurons were susceptible to cell loss and accumulation of neurofibrillary tangles in entorhinal cortex (EC) and area CA1 (Braak & Braak, 1991; Giannakopoulos et al., 1995; Gómez-Isla et al., 1996; Hyman et al., 1984). Layer II of EC gives rise to the perforant pathway, which is the primary source of cortical-hippocampal

innervation. Therefore, cell death and pathology in layer II of EC, reported in multiple studies of postmortem AD brains, suggests compromised hippocampal circuitry.

The PFC is another region implicated in AD progression, although neuropathology in this region appears later than it does in the hippocampus (Braak & Braak, 1991). The PFC communicates with limbic structures, including the hippocampus, that are affected earlier in disease progression (Jay et al., 1989; Verwer et al., 1997), and is susceptible to age-related declines in gray matter (Raz et al., 1997). Recent work suggests disrupted connectivity between the hippocampus and PFC in AD patients (L. Wang et al., 2006), implicating broader network dysfunction in AD patients. Importantly, gene expression analysis from frontal cortices of AD patients revealed unique clusters of gene expression changes that preceded AD-associated pathological hallmarks, suggesting that such gene targets may be well-suited to early interventional treatments for AD (Bossers et al., 2010). Newly emerging evidence for gene expression changes that occur prior to plaque deposition and tau accumulation suggests that non-amyloid and tau-targeted therapeutics, and a focus instead on modulating gene expression, may hold promise for treatment of AD.

APOE and Alzheimer's disease

Genetic factors contribute to both early and late-onset AD. Although early-onset AD has a higher instance of heritability, late-onset AD, which represents the majority of AD cases, is associated with *APOE4* genotype as its predominant genetic risk factor (Ertekin-Taner, 2007; van der Flier et al., 2011; Yu et al., 2014). The *APOE* gene codes for the

protein apolipoprotein E, which regulates cholesterol transport in the brain (Leduc et al., 2010; Poirier, 2005; Weisgraber & Mahley, 1996). Although the estimated prevalence of allelic differences in *APOE* differs by ethnicity and geographic location, researchers estimate that approximately 77.9% of the worldwide population carries the *APOE3* allele, whereas the *APOE4* and *APOE2* alleles are significantly less prevalent, occurring at an incidence of 13.7% and 8.4%, respectively (Farrer et al., 1997; Ferri et al., 2005).

Strikingly, the *APOE4* genotype contributes to up to 15-fold greater risk of AD than the more common *APOE3* genotype, and even a single copy of the *APOE4* allele can magnify risk by 2-fold (Bertram, 2009; Roses, 1996). Furthermore, over 50% of AD patients carry the *APOE4* allele, whereas only 15% of healthy controls do so (Ward et al., 2012). In addition to increasing risk for AD, *APOE4* also appears to contribute to earlier disease onset (Corder et al., 1993) and earlier senile plaque accumulation (Kok et al., 2009). Single and double copies of *APOE4* also increased the rate of cognitive decline in AD patients relative to patients carrying other *APOE* alleles (Craft et al., 1998; Qian et al., 2021). Although it is well established that *APOE4* worsens disease outcomes and conveys increased risk for AD relative to other genotypes, the mechanisms through which it does so remain poorly understood. Although not all AD cases are linked to *APOE4* genotype, understanding the root of *APOE4*-associated disease risk would be of great benefit to a large portion of individuals affected by AD.

Female sex as a risk factor for AD

Sex is another major risk factor for AD, such that risk for women of developing AD is significantly greater than that of men (Nebel et al., 2018). Indeed, women represent two thirds of AD cases in individuals over age 65 (Andersen et al., 1999; Launer et al., 1999). This difference in disease prevalence is sometimes attributed to age alone, given women's longer life expectancy. However, studies controlling for age find disparities between sexes that suggest higher disease prevalence among women compared to men. For instance, even when gender-dependent mortality rates are accounted for, women are at a two-fold risk of developing AD compared to men (Carter et al., 2012; Viña & Lloret, 2010). Moreover, even at age of 45, the lifetime risk for AD is one in five for women and one in ten for men (Chêne et al., 2015).

Female sex also contributes to worse cognitive decline relative to male sex in AD patients. A meta-analysis of several studies including neurocognitive data from patients with AD revealed that men performed better than their women counterparts on verbal and visuospatial tasks as well as tests of episodic and semantic memory (Irvine et al., 2012). These data are consistent with other findings that female sex exacerbates cognitive decline relative to male sex in AD patients, in one instance to a similar magnitude as *APOE4* genotype alone (Holland et al., 2013). Furthermore, the relationship between global AD pathology and clinical diagnosis is stronger in women than in men (Barnes et al., 2005), suggesting that AD pathology may be more closely linked to dementia in women than in men. Thus, examining the contributions of sex and gender to AD etiology is critical for our overall understanding of the disease.

APOE and sex interactions

Perhaps unsurprisingly, sex and *APOE* genotype interact to promote risk for AD. Female *APOE4* carriers are more likely than male *APOE4* carriers to develop AD, suggesting a sex by genotype interaction (Altmann et al., 2014; Bretsky et al., 1999; Farrer et al., 1997; Kim et al., 2009). In support, *APOE* genotype correlates strongly with pathological and behavioral hallmarks of AD, more so in women than in men. For example, levels of total and phosphorylated tau protein in cerebrospinal fluid were more strongly associated with *APOE4* genotype in women carriers than in male carriers (Altmann et al., 2014; Hohman et al., 2018). *APOE4* was also coupled more strongly to cognitive decline in women than in men (Hobel et al., 2019; Mortensen & Høgh, 2001). Indeed, two copies of *APOE4* conferred a larger negative impact on women than on men, resulting in poorer global cognition between the ages of 70-74, and worse memory between the ages of 65-69 (Hobel et al., 2019). Ultimately, understanding why *APOE4* genotype is particularly detrimental to women carriers is of central importance to developing new approaches for AD prevention and treatment.

Molecular correlates of Alzheimer's disease

Studying the molecular correlates of AD is uniquely challenging. Both normal cognitive aging and pathological aging lead to cognitive decline. Moreover, many other dementia-causing illnesses exist, multiple of which are also characterized by misfolded proteins as in AD (Agorogiannis et al., 2004; Sweeney et al., 2017). Thus, differentiating AD-associated mechanisms from normal aging or other dementias can be difficult. Significant

progress has been made with the advent of imaging approaches such as amyloid and tau PET, and ongoing searches for early biomarkers of disease will hopefully yield additional methods of early diagnosis and intervention. Despite the challenges associated with identifying molecular correlates specific to AD, researchers have made significant progress towards understanding what molecular changes occur in the brains of individuals with AD, mostly via postmortem tissue analyses.

Synaptic dysfunction is a principal component of AD pathology that underlies AD-associated cognitive decline (DeKosky & Scheff, 1990; LaFerla & Oddo, 2005). AD patients experience both pre- and post-synaptic protein loss, particularly in the neocortex and hippocampus, brain regions selectively vulnerable to both AD pathology and neuronal loss (Masliah et al., 2001; Reddy et al., 2005). Increased synaptic protein loss has been linked to *APOE4* genotype (Tannenberg et al., 2006), and decreased dendritic spine density in *APOE4+* individuals mirrors similar decreases in synaptic proteins (Dumanis et al., 2009; Ji et al., 2003). Because excitatory signaling is thought to be localized to dendritic spines, AD-related reductions in dendritic arborization and spine density substantially diminish excitatory synaptic plasticity and impair cognition (Herms & Dorostkar, 2016). However, few studies have directly compared the influences of sex and *APOE4* genotype in combination on synaptic protein levels and spine morphology. Indeed, the influence of sex alone on synaptic protein levels and spine morphology in AD patients and mouse models remains unclear, although existing studies suggest synaptic integrity is reduced in females (Jiao et al., 2016; Rijpma et al., 2013). Thus, whether the negative impact of *APOE4* on synaptic integrity and dendritic spine density is exacerbated by female sex remains unclear.

Mitogen associated protein kinase (MAPK) signaling through phosphorylated extracellular signal regulated kinase (pERK) facilitates hippocampus-dependent learning and memory (Atkins et al., 1998; Selcher et al., 1999), making it an appealing target for cognitive therapeutics targeting memory dysfunction. Moreover, MAPK signaling interfaces with many other signaling cascades such as phosphatidylinositol 3-kinase (PI3K) and c-Jun N-terminal kinase (JNK) within the hippocampus to enhance memory consolidation in both males and females (Koss & Frick, 2017; Sherrin et al., 2011). MAPK signaling dysfunction has also been linked to AD, although it has received comparatively little attention within the field of AD research relative to other molecular events (Pei et al., 2002). Previous work indicates that MAPK signaling is differentially affected by *APOE* genotype (Salomon-Zimri et al., 2019) and by sex (Koss et al., 2018). Elucidating whether these memory-related signaling pathways are modulated by *APOE* genotype and sex may yield important insights into the pathogenesis of memory dysfunction in AD.

In addition to aberrant synapse and cell signaling, neuroinflammation has long been observed to be an early symptom in AD pathology, driven by both astrogliosis and microgliosis (Heneka et al., 2015). Importantly, female mice have significantly higher numbers of both astrocytes and microglia relative to male mice, and this cell population increases with age (Mouton et al., 2002). Moreover, microglia-specific gene expression markedly differs by sex in the aged mouse hippocampus (Mangold et al., 2017). Yet, as with synaptic integrity and intracellular signaling, little is known about how glial activation in the AD brain is modulated by female sex and *APOE4* genotype in concert.

Estrogens as a modulator of *APOE*-associated disease risk

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 17-β 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 & Henderson, 1996). In fact, female AD patients have lower levels of endogenous E_2 than age-matched controls (Manly et al., 2000; Tsolaki et al., 2005). Just as low levels of circulating E_2 are tied to cognitive decline, treatment with exogenous E_2 is often linked to better health outcomes. Studies including female subjects taking estrogen replacement therapy, although somewhat controversial, offer support for the idea that E_2 may be beneficial for AD patients depending on timing of initiation and treatment regimen (Henderson, 2006). For example, risk for developing AD was reduced in individuals undergoing estrogen replacement therapy, an effect that was magnified when dosage and length of treatment increased (Paganini-Hill & Henderson, 1996).

Importantly, female *APOE4* carriers seem less receptive to the beneficial effects of E_2 relative to *APOE4*-negative counterparts (Yaffe et al., 2000). Clinical work is consistent with evidence from basic research; *APOE2* and *APOE3*, but not *APOE4*, acted 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.

17β-estradiol in learning and memory

In normal, nonpathological states, E_2 can facilitate cognitive processes. In healthy adult mice, treatment with exogenous E_2 facilitates memory via rapid activation of cell signaling within the dorsal hippocampus (DH), thereby increasing protein expression of phosphorylated proteins such as extracellular signal-regulated kinase and phosphatidylinositol 3-kinase, eukaryotic initiation factor 4E binding protein , cAMP-response element binding protein, and other memory-related molecules (Boulware et al., 2013; Fernandez et al., 2008; Fortress, Fan, et al., 2013; Tuscher, Luine, et al., 2016). These cell-signaling events driven by E_2 treatment are accompanied by an increase in dendritic spine density in the DH and in the medial prefrontal cortex (Tuscher, Luine, et al., 2016). However, whether a similar treatment regimen of E_2 can facilitate memory, activate cell-signaling cascades, and increase dendritic spine density in a model of AD is unknown. Furthermore, whether exogenous E_2 may interact with *APOE* in a genotype-specific manner to modulate memory and its associated neural correlates remains an open question.

The EFAD model

Despite evidence implicating the interaction between *APOE* genotype and sex as a crucial determinant of AD risk, the intersection of genotype and sex is frequently overlooked. Transgenic (Tg) mice are an important tool to leverage towards establishing the basic neurobiological underpinnings of factors, such as genotype and sex, associated with increased AD risk. EFAD-Tg mice express 5 familial AD mutations (5xFAD) that

contribute to accumulation of Aβ42, and express human *APOE3* (E3FAD) or *APOE4* (E4FAD), making them ideal subjects for investigating the relative contributions of *APOE* genotype and sex (Tai et al., 2017; Youmans et al., 2012). Targeted replacement of murine *APOE* with human *APOE* is a key feature of this model (Youmans et al., 2012), given significant structural and functional differences between the two (Fagan et al., 2002). The EFAD model recapitulates human clinical presentation of *APOE*-associated AD, such that female mice homozygous for *APOE4* (E4FAD) exhibit earlier onset of, and more advanced, disease pathology relative to other groups (Stephen et al., 2019; Thomas et al., 2016). E4FAD females also have the shortest lifespan among the EFAD allelic variants, followed by E4FAD males and E3FAD females, and finally by E3FAD males (Balu et al., 2019). Given the aforementioned data suggesting that the EFAD model appropriately stratifies sex and genotype differences, the EFAD mouse model is uniquely suited for investigation of genotype and sex differences in concert.

Summary and significance

Female sex and *APOE* genotype act synergistically to increase AD risk and severity, an effect that can be modeled with EFAD mice (Tai et al., 2017; Youmans et al., 2012). Furthermore, although infusion of E_2 into the dorsal hippocampus of young adult mice can enhance object memory via cell signaling activation and increases in dendritic spine density, whether intrahippocampal E_2 is of mnemonic benefit in mice with AD-like pathology remains unclear. Therefore, the overall objective of the dissertation was to elucidate the interactions among sex, *APOE* genotype, and E_2 in a mouse model of AD

meant to recapitulate *APOE*-associated disease risk, and their relative influence on memory and anxiety-like behaviors, protein expression, and dendritic spine density. In the first experiment of this dissertation, we tested whether *APOE4* genotype and female sex interacted to adversely affect memory consolidation and anxiety-like behavior in the EFAD model. We also asked whether *APOE4* genotype and female sex interacted to negatively influence protein expression in the DH, and dendritic spine density and morphology in the DH and mPFC. The methods, results, and implications of this experiment are discussed in Chapter 2. In the second experiment of this dissertation, we tested whether *APOE4* genotype and gonadal status modulate anxiety-like behavior in the open field in female EFADs. Chapter 3 covers methods, results, and discussion of this second experiment. Lastly, in the third experiment of this dissertation, we tested whether *APOE4* genotype prevented the memory-enhancing effects of E_2. This final experiment comprises Chapter 4 of the dissertation. Our long-term goal is to identify molecular mechanisms that underly *APOE4*-associated cognitive dysfunction, and that may be differentially and concurrently affected by sex and E_2. Chapter 5 summarizes the broad implications of the results from the dissertation and suggests future directions.

CHAPTER TWO: Defining the impact of *APOE* genotype on sex differences in memory formation and neural function in EFAD mice

The primary objective of this experiment was to determine whether *APOE* genotype and sex act independently or together to modulate object memory consolidation, protein expression, and dendritic spine density and morphology. Although *APOE4* genotype and female sex contribute to AD risk both independently and together (Altmann et al., 2014; Bretsky et al., 1999; Farrer et al., 1997), the factors underlying the relationship between these two variables are poorly understood.

In existing rodent models of AD, disease severity is disproportionately exacerbated in females relative to males, as reflected by worsened neuropathological and cognitive outcomes (Schmid et al., 2019; Wang et al., 2003; Yue et al., 2011). Likewise, in rodent models designed to interrogate the impact of *APOE* status on cognitive function and neural correlates of AD, either through knockout of *APOE* or targeted replacement of murine *APOE* with human *APOE*, *APOE4* has a deleterious effect on cognitive capacity, dendritic spine density, neurogenesis, and synaptic integrity (Ji et al., 2003; Koutseff et al., 2014; Wang et al., 2005). However, *APOE* genotype and sex are rarely accounted for in concert in studies using animal models of AD to investigate molecular and behavioral disease correlates. Thus, the EFAD model is uniquely suited for interrogating the ways in which female sex and *APOE4* genotype interact against a background of AD-like pathology. Because cell signaling dysregulation and neuroanatomical changes have been implicated in the pathogenesis of AD (Herms & Dorostkar, 2016; Pei et al., 2002), asking how female sex and *APOE4* genotype modulate protein expression and dendritic spine density could provide novel insight into mechanisms contributing to individual

differences in disease severity. Our working hypothesis was that female *APOE4*-expressing EFAD mice would exhibit impaired memory consolidation, protein expression, and decreased dendritic spine density and morphology relative to other groups.

Materials and Methods

Subjects

Male and female EFAD (APOE+/+/5xFAD+/-) mice co-express five familial AD mutations (APP K670N/M671L + I716V + V717I and PS1 M146L + L286V) under control of the neuron-specific mouse Thy-1 promoter, and are homozygous for human APOE3 or APOE4 (Youmans et al., 2012). EFAD mice were bred, weaned, and genotyped at the University of Illinois Chicago (UIC; Animal use protocol 17-066) before shipment to the University of Wisconsin-Milwaukee (UWM) at 2 months (M) of age, where they were aged to 6M before the start of behavioral testing (animal use protocol 19-20-03). At both UIC and UWM mice were housed in groups of up to 5 per cage and maintained on a 12 hours (h) light/dark cycle with ad libitum access to food and water. Mice were received from UIC and behaviorally tested in two separate cohorts, whereas brain analyses for all mice were conducted at the same time. Procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the UIC Animal Care Committee and UWM Institutional Animal Care and Use Committee.

General Experimental Design

At 6M, male and female mice were tested in a battery of commonly used hippocampus-dependent memory tasks including object recognition (OR), object placement (OP), and the Morris Water Maze (MWM) (Fig. 1). The order of OR and OP testing was counter-balanced across groups such that half of the mice underwent OR first, whereas the other half underwent OP first to eliminate any order-of-testing effects. Mice were then tested in the MWM. Two weeks after the completion of all behavioral testing, brains were extracted and hemisected for Golgi analysis and Western blotting experiments. The dorsal hippocampus was immediately dissected from the whole brain or from the left hemisphere and frozen at -80°C for Western blotting (n= 9-16/group), and right hemispheres were collected for Golgi staining and morphological analysis (n = 5-7/group). These sample sizes are sufficiently powered to detect between group differences.

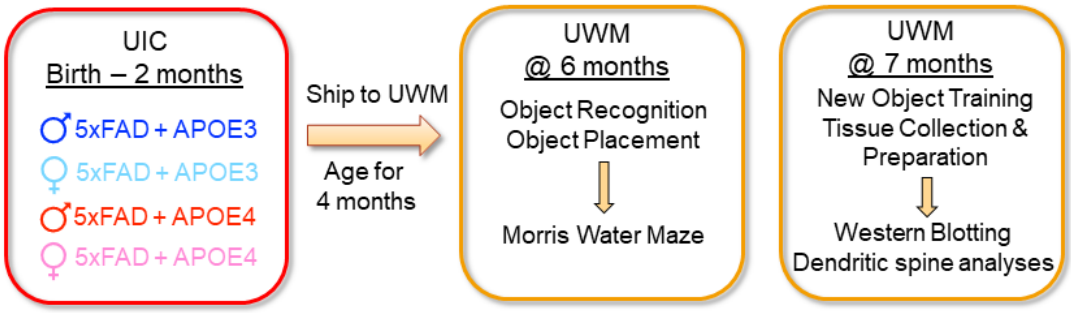


Fig. 1. Experimental timeline. Mice were bred at the University of Illinois Chicago (UIC) and were then sent to the University of Wisconsin-Milwaukee (UWM) at 8 weeks of age, where they were aged for four months prior to the start of testing. At 6 months of age, mice were tested in the object recognition and object placement tasks, followed by Morris water maze testing. At 7 months of age, brain tissue was collected and analyzed.

Behavioral testing

Object Recognition (OR) and Object Placement (OP)

To determine whether sex differentially or synergistically with *APOE3* or *APOE4* genotype influences memory for a previously seen object or location, object recognition and spatial memory were tested in male and female E3FAD and E4FAD mice (n = 12-16/group) using the OR and OP tasks, as described previously (Boulware et al., 2013; Fernandez et al., 2008; Fortress, Schram, et al., 2013; Kim et al., 2016; Koss et al., 2018; Taxier et al., 2019; Tuscher, Szinte, et al., 2016). Briefly, mice were handled for 30 seconds (s)/day for 3 days and then habituated to the testing arena for 5 minutes (m)/day for 2 consecutive days. On the training day, mice were returned to the empty arena for 2m, and then were removed to a holding cage, during which time 2 identical objects were placed 5cm from the upper left and right corners of the arena. Upon their return to the arena, mice freely interacted with the objects until they accumulated 30s of object exploration (or until 20m elapsed). Mice were then returned to their home cage for 24h or 4h to await testing in OR and OP, respectively; wild-type mice exhibit intact memory for the identity (OR) and location (OP) of objects at these timepoints (Boulware et al., 2013; Fortress, Schram, et al., 2013; Kim et al., 2016).

During OR testing, one familiar training object was replaced with a novel object. Mice again freely interacted with the objects until they accumulated 30s of object exploration. Mice that remember the familiar training object spend more time than chance (15s) with the novel object during testing. During OP testing, one training object was moved to a new location (bottom left or right corner). Mice that remember the location of the training

objects spend more time than chance (15s) with the moved object during testing. Time spent with the objects and time to accumulate 30s were recorded using ANYmaze automated tracking software (San Diego Instruments).

Morris Water Maze

Spatial memory was also tested using the MWM, as described previously (Benoit et al., 2015; Harburger et al., 2007). Data were recorded using ANYmaze software. A 4-trial shaping procedure was conducted one day prior to testing to acclimate the mice to escaping onto a 10x10 cm platform. Spatial memory was then tested in 5 consecutive daily sessions consisting of 6 trials/day. The first 5 trials were hidden platform trials during which the escape platform was located 1.5 cm below the water's surface. Mice were allowed 60s to find the platform. The sixth trial was a 60s probe trial in which memory was tested in the absence of the platform, which was lowered for the first 30s, then raised and available for escape for the remainder of the trial to discourage extinction of escape behavior. The platform remained in the same place throughout testing and the intertrial interval was 10-20m. During hidden platform trials, swim time (s), swim distance to platform location during the first trial (cm), and swim speed (cm/s) were recorded using ANYmaze. Swim distance to platform location during the first trial (cm) was also recorded as a measure of retention of learning from the previous day's acquisition trials. During probe trials, the % time in the correct quadrant (containing the platform), time to first entry into the correct quadrant (s), average distance from the platform (cm), and # of platform crossings during the first 30s of the probe trial were measured.

One day after spatial testing, mice were tested in a cued version of the task in which the escape platform was raised above the surface of the water and made visible with red tape. Because memory is not necessary to locate the platform, this task assesses motivation, visual ability, and swimming ability. Six trials/day were conducted for 3 consecutive days during which the platform location changed for each trial. Swim time, swim distance, and swim speed were recorded.

Western blotting

Western blotting was used to determine the extent to which sex and *APOE* genotype alter the expression of several categories of proteins in the dorsal hippocampus 5m after training with novel objects. This brain region is of particular interest due to abundant evidence that integrity of hippocampal function is compromised in AD, and previous work using the EFAD model suggesting that sex and *APOE* genotype may modulate protein expression within this brain region (Liu et al., 2015).

Western blotting was conducted as described previously (Boulware et al., 2013; Fernandez et al., 2008; Fortress, Schram, et al., 2013; Kim et al., 2016; Koss et al., 2018; Taxier et al., 2019; Zhao et al., 2012). Tissue samples were suspended 1:25 w/v in hypotonic lysis buffer and homogenized. Homogenates were electrophoresed on 10% TGX (Tris-Glycine eXtended) stain-free precast gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes; protein transfer was verified using a ChemiDoc MP gel imager (Bio-Rad). After blocking in 5-8% milk, membranes were incubated overnight at 4°C in primary antibodies. The following day, blots were incubated at room temperature

with the appropriate secondary antibody. Blots were developed using Clarity Max chemiluminescent substrate (Bio-Rad) and protein expression detected using a ChemiDoc MP gel imager. Densitometry was performed using Image Lab software (Bio-Rad Image Lab v 5.2). Phosphorylated proteins were normalized to their respective total proteins. The remaining 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 male E3FADs.

Golgi Impregnation and Analyses

Left hemispheres (n=5-7/group) were collected for Golgi impregnation, which was performed as described previously (Frankfurt et al., 2011; Kim et al., 2019; Tuscher, Luine, et al., 2016) using the Rapid Golgi Stain Kit (FD Neuro Technologies). Tissue was sliced into 100μm sections and mounted on gelatin-coated microscope slides, which were coded so that the individual counting spines was blind to treatment.

Secondary basal dendrites and tertiary apical dendrites were counted from pyramidal neurons in the dorsal hippocampal CA1 and layer II/III of the mPFC under an Olympus BX51WI microscope (100x with oil) using NeuroLucida (v 11.08, MBF Bioscience). Selected dendrites were between 10-20μm in length and 0.5-1.3μm thick. Neurons selected for analysis were required to have well impregnated cell bodies and dendrites, and had to be clearly distinguishable from adjacent cells. Two dendritic segments/neuron and 6 cells/region were included in the analysis. Spine density was calculated as the number of spines/10μm dendrite.

Dendritic spines were identified according to three categories based on shape: mushroom, stubby, or thin (Harris et al., 1992; Kim et al., 2019). Mushroom spines had head diameters at least twice the size of their neck diameters, whereas stubby spines had neck diameters relatively equal to the total length of the spine. Thin spines had head diameters less than or equal to their neck diameters.

CA1 dendritic complexity was quantified in using Sholl analysis under an Olympus BX51WI microscope (100x with oil). Cell bodies, apical dendrites, and basal dendrites were traced using NeuroLucida (v 11.08, MBF Bioscience). Neurons selected for tracing had to be present within the middle thickness of the section, be fully impregnated, and have at least 3 primary basilar dendrites, each of which needed to branch at least once. Concentric spheres were used to count the number of intersections made by dendritic branches at successive 10μm steps from the cell body. The number of intersections made by dendritic branches at increasing diameters from the soma served as a measure of dendritic complexity (Sholl, 1953). Sholl analysis was attempted in the mPFC but was not completed because Golgi stain penetrance was not as robust as in CA1, and therefore was not reflective of full branching complexity.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 9. For OR and OP, one-sample *t* tests were used to determine whether the time spent with each object differed from chance (15s), which assesses within-group learning. Between-group differences were assessed using two-way ANOVAs with sex and genotype as between-subject

variables. Similar two-way ANOVAs were used to analyze Western blot and dendritic spine density data. For the MWM, two-way repeated measures ANOVAs, with sex and genotype as between subject variables, and session as the repeated measure, were conducted to evaluate within and between-group differences in learning across days. For Sholl analysis, two-way repeated measures ANOVAs, with sex and genotype as between subject variables and distance from the cell soma as the repeated measure, were conducted to evaluate within and between-group differences in dendritic complexity. Significant main effects were followed by planned comparisons where appropriate, given our a priori hypothesis that deficits would be greatest in female E4FAD mice, followed by E4FAD males, E3FAD females, and then E3FAD males. Statistical significance was set at $p < 0.05$ for all statistical tests, and trends were determined by $p < 0.10$.

Results

Object Recognition

Male E3FADs spent significantly more time than chance (15s) with the novel object during testing (Fig. 2A; $t_{(15)} = 5.35$, $p < 0.0001$; n=12-16/group), indicating intact memory for the identity of the training objects. By contrast, female E3FADs and E4FADs of both sexes did not spend significantly more time than chance with the novel object during testing, suggesting impaired object recognition memory among all but the male E3FAD group. E3FADs spent more time with the novel object during testing than E4FADs ($F_{(1, 55)} = 5.82$, $p = 0.02$), an effect driven by male E3FADs, which spent more time with the novel object than E4FADs of both sexes ($p = 0.01$). No other main effects or interactions were

significant. Time to accumulate 30s of object exploration did not differ among male E3FADs (M = 461.68, SEM = 51.0), female E3FADs (M = 443.89, SEM = 49.44), male E4FADs (M = 508.63, SEM = 73.06), or female E4FADs (M = 545.76, SEM = 74.39), suggesting that total activity or motivation to explore objects was not impacted by sex or *APOE* genotype.

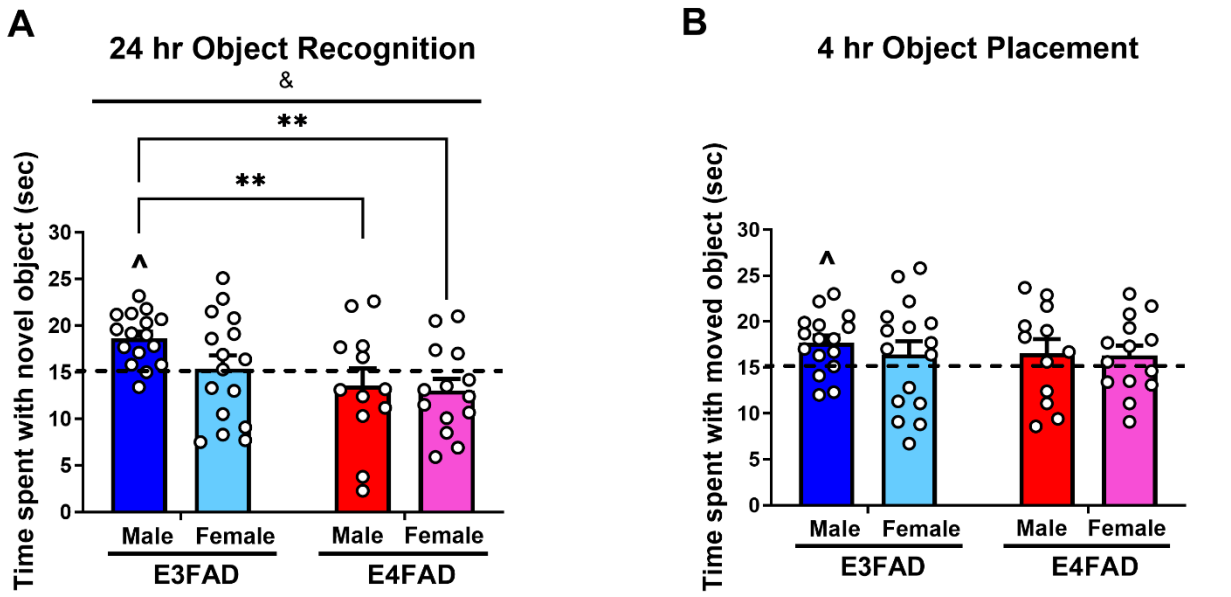


Fig. 2. All mice but male E3FADs displayed impaired object recognition and spatial memory formation. Only male E3FADs exhibited intact memory for the identity (A) and location (B) of the training objects. Male E3FADs spent significantly more time than chance (dashed line at 15 sec; ^$p < 0.05$) with the novel (A) and moved (B) objects during testing. E3FADs spent more time than E4FADs with the novel object ($^{\&}p < 0.05$ = main effect of genotype), and male E3FADs spent significantly more time than E4FADs of either sex with the novel object (**$p < 0.01$). Bars represent mean ± standard error of the mean (SEM).

Object Placement

Similar results were observed for OP. Male E3FADs spent significantly more time than chance with the moved object during testing (Fig. 2B; $t_{(15)} = 3.360$, $p = 0.0043$; n=12-

16/group), suggesting intact memory for the location of the training objects. As in OR, female E3FADs and E4FADs of both sexes did not spend more time than chance with the moved object during testing. Neither the main effects nor interaction were significant for OP. Again, time to accumulate 30s of object exploration did not differ among male E3FADs (M = 407.66, SEM = 33.53), female E3FADs (M = 363.61, SEM = 37.63), male E4FADs (M = 436.12, SEM = 31.24), or female E4FADs (M = 426.46, SEM = 53.6).

Together, within-subjects t-tests for OR and OP indicate that only male E3FADs exhibited intact memory for previously learned objects or locations, and suggest both a detrimental effect of *APOE4* genotype on object recognition and spatial memory, as well as an interesting sex difference favoring males within the *APOE3* genotype.

Morris Water Maze

All ANOVA statistics for each MWM measure are presented in Table 1 (n=14-16/group for all measures). The sections below summarize the main effects and interactions, and present results of planned comparisons where appropriate.

Two male E4FADs and two female E4FADs were excluded from all water maze analyses because their average swim speed was greater than two standard deviations away from their respective group's average swim speed in visible platform trials. All four excluded mice also spent time immobile (i.e., floating) during the task, suggesting that they were not motivated to search for the hidden platform.

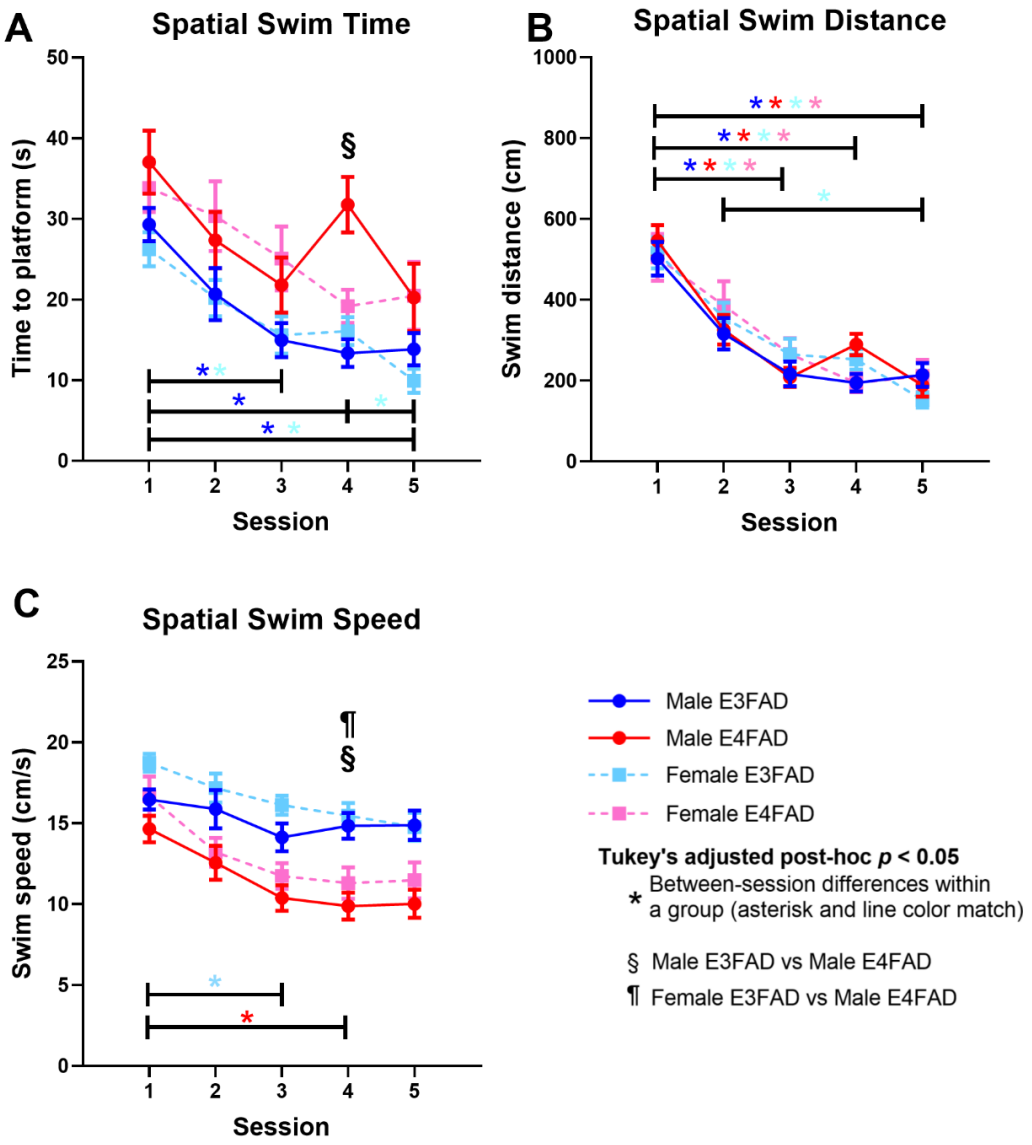
Table 1. ANOVA statistics for Morris Water Maze variables

Task	Variable	Sex	Genotype	Sex x Genotype	Session	Session x Sex	Session x Genotype	Session x Sex x Genotype
Spatial	Swim time	F(1,54)=0.53	F(1,54)=22.42, *p*<0.0001	F(1,54)=0.08	F(3.48,187.9)=23.51, *p*<0.0001	F(4,216)=1.39	F(4,216)=0.23	F(4,216)=2.71, *p*<0.03
	Swim distance	F(1,54)=0.24	F(1,54)=0.42	F(1,54)=0.20	F(3.19,172.2)=61.3, *p*<0.0001	F(4,216)=1.25	F(4,216)=0.09	F(4,216)=1.78
	Swim distance, 1st trial	F(1,54)=2.36	*F(1,54)=3.62, p=0.63*	F(1,54)=0.79	F(2.88,155.7)=31.57, *p*<0.0001	F(4,216)=1.38	*F(4,216)=2.36, p=0.05*	F(4,216)=1.64
	Swim speed	F(1,54)=4.38, *p*=0.04	F(1,54)=34.72, *p*<0.0001	F(1,54)=0.017	F(3.21,173.1)=21.54, *p*<0.0001	F(4,216)=0.7	F(4,216)=2.01	F(4,216)=0.47
	Quadrant time	F(1,53)=2.78	F(1,53)=0.0007	F(1,53)=4.16, *p*=0.046	F(3.46,183.2)=23.64, *p*<0.0001	F(4,212)=2.05	F(4,212)=3.48, *p*<0.009	F(4,212)=1.25
	Avg distance	F(1,54)=1.42	F(1,54)=0.33	F(1,54)=2.85, *p*=0.1	F(3.36,181.2)=36.22, *p*<0.0001	F(4,216)=2.4, p=0.05	F(4,216)=3.1, *p*<0.02	F(4,216)=1.47
	Time to 1st entry	F(1,54)=0.58	F(1,54)=2.26	F(1,54)=4.7, *p*=0.03	F(3.643,196.7)=4.07, *p*<0.046	F(4,216)=1.19	F(4,216)=0.45	F(4,216)=1.12
	Platform crossings	F(1,54)=0.29	F(1,54)=1.98	F(1,54)=5.46, *p*<0.02	F(3.74, 201.9)=4.089, *p*<0.005	F(4,216)=0.25	F(4,216)=0.42	F(4,216)=0.89
Cued	Swim time	F(1,54)=0.003	F(1,54)=0.78	F(1,54)=3.56, *p*<0.065	F(1.69,90.96)=56.98, *p*<0.0001	F(2,108)=0.15	F(2,108)=0.36	F(2,108)=0.23
	Swim distance	F(1,53)=6.408e-005	F(1,53)=0.03	F(1,53)=1.96	F(1.65,87.54)=6.01, *p*<0.006	F(2,106)=0.06	F(2,106)=0.69	F(2,106)=0.46
	Swim speed	F(1,54)=0.52	F(1,54)=0.68	F(1,54)=0.27	F(1.46,78.90)=19.97, *p*<0.0001	F(2,108)=3.45, *p*<0.04	F(2,108)=0.95	F(2,108)=0.06

Spatial learning trials

Mice of both genotypes could learn the location of the hidden platform, as indicated by significant main effects of session (Table 1) for swim time (Fig. 3A), cumulative swim distance (Fig. 3B), and swim speed (Fig. 3C). The main effect of genotype was significant for swim time and swim speed, such that E3FADs swam faster and located the platform in less time than E4FADs. The main effect of genotype was not significant for swim distance, suggesting that the genotype effect in swim time may be due primarily to differences in swim speed. The main effect of sex was significant only for swim speed, in which females swam faster than males.

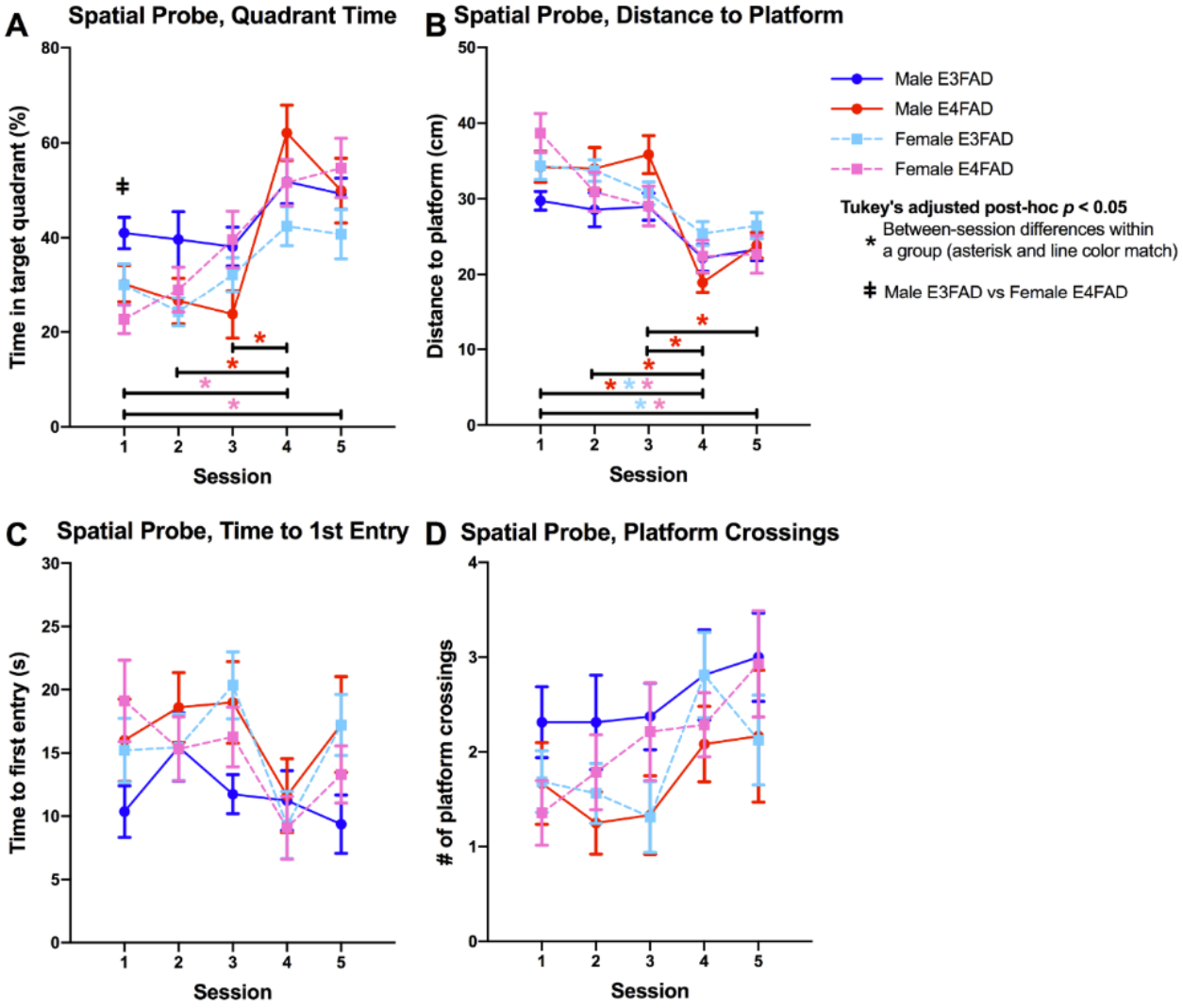
Sex and genotype effects varied across sessions, as indicated by a significant session x sex x genotype effect for swim time and session x genotype effect for swim distance in the first trial. Indeed, by sessions 3 (halfway through testing) and 5 (end of testing), E3FADs of both sexes reached the platform in less time than during session 1 ($p < 0.05$), whereas E4FADs of both sexes did not (Fig. 3A), suggesting that E3FADs of both sexes learned the platform location more rapidly than E4FADs. In addition, the swim times of E3FAD males were significantly faster than E4FAD males in session 4 ($p = 0.02$). Male E4FADs also had a significantly and unusually higher spatial swim times than other groups during session 4, potentially driven by their slower swim speed. Overall, these data suggest minimal effects of sex or genotype on learning-related parameters (e.g., spatial swim time or spatial swim distance) associated with Morris water maze task acquisition.



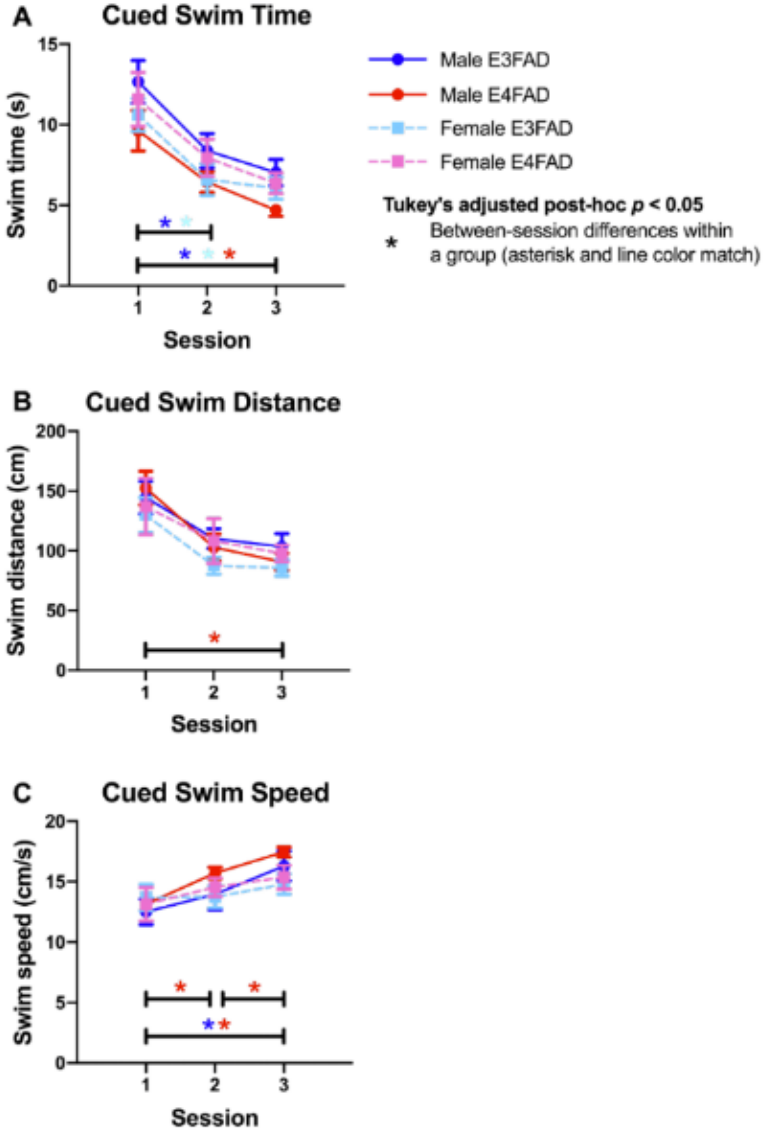
<u>Fig. 3</u>. All groups learned the platform location in the hidden platform trials of the Morris Water Maze. Spatial swim time (A), swim distance (B), and swim speed (C) decreased across sessions in all groups. Differences between sessions within a group are indicated with asterisks that match the group's line/symbol color (*$p < 0.05$; dark blue = male E3FAD, light blue = female E3FAD, red = male E4FAD, pink = female E4FAD). Between-group differences are indicated above the session during which they occurred (§ = male E3FAD vs male E4FAD; ¶ = female E3FAD vs male E4FAD). Symbols represent the mean ± SEM.

Probe trials

During the first 30s of each daily probe trial, the platform was submerged and inaccessible for escape. As in the spatial learning trials, significant session effects for the percent time spent in the target quadrant (quadrant time; Fig. 4A), average distance to the platform (distance to platform; Fig. 4B), time taken to first enter the location of the platform (time to first entry; Fig. 4C), and platform crossings (Fig. 4D) indicated that all groups learned the location of the hidden platform. Effects of sex were limited to a single sex x session interaction for distance to the platform and sex x genotype interactions present in all measures but distance to the platform (although there was a weak trend of $p = 0.097$). Effects of genotype also varied by session, as indicated by session x genotype interactions for quadrant time and distance to the platform. Performance in all measures tended to be quite variable, making definitive conclusions about effects of sex and genotype difficult to draw. In general, male E3FADs tended to outperform other groups in sessions 1-3, where they spent more time in the target quadrant, swam shorter distances to the platform, entered the platform area faster, and made more platform crossings than other groups. One notable between-group difference was observed in quadrant time, where male E3FADs spent more time in the target quadrant than female E4FADs during session 1 (Fig. 4A; $p < 0.03$). E3FAD males also tended to outperform E4FAD males in sessions 1-3. Although subtle sex and genotype interactions were observed in within-group performance, the overall variability and lack of main effects of sex or genotype on probe trial measures suggests that neither sex nor genotype significantly modulated memory expressed in the probe trials of this task.



<u>Fig. 4</u> Spatial memory in the probe trials of the Morris water maze was similar in all groups. Percent time in target quadrant (A) and number of platform crossings (D) increaesed across sessions. Distance to the platform (B) and latency to enter the platform's location (C) decreased across sessions. Differences between sessions within a group are indicated with asterisks that match the group's line/symbol color (* $p < 0.05$; dark blue = male E3FAD, light blue = female E3FAD; red = male E4FAD, pink = female E4FAD) One between-group difference is indicated above the session during which it occurred (‡ = male E3FAD vs female E4FAD). Symbols represent the mean ± SEM.



<u>Fig. 5</u> Performance in the cued (visible platform) trials of the Morris water maze did not differ among groups. Swim time (A) and swim distance (B) decreased across sessions, whereas swim speed (C) increased. Between time point differences within strains are color matched (* p < 0.05; dark blue = male E3FAD, light blue = female E3FAD; red = male E4FAD, pink = female E4FAD). Symbols represent the mean ± SEM.

Cued trials

All groups learned to find the visible platform, as indicated by main effects of session for cued swim time (Fig. 5A), swim distance (Fig. 5B), and swim speed (Fig. 5C). No effects of genotype were observed in any measure, and the sole sex effect was a sex x session interaction for swim speed, reflecting faster speeds in male E4FADs in sessions 2 and 3 compared to session 1 ($p < 0.001$). These data suggest that EFAD mice of either sex or genotype can learn to locate and swim to a visible platform, indicating no adverse effects of sex or genotype on sensorimotor abilities, motivation, or swimming ability.

Western Blotting

Synaptic proteins

E3FADs of both sexes exhibited higher levels of PSD95 (Fig. 6A; $F_{(1, 50)}$ = 6.24 , p = 0.02) and synaptophysin (Fig. 6B; $F_{(1, 50)}$ = 5.17, p = 0.03) than E4FADs of both sexes (n=14/group). PSD95 levels were higher in E3FAD females than E4FAD females (p = 0.03). Combined, these data suggest a detrimental effect of *APOE4* genotype on synaptic proteins, perhaps indicating a reduction in synapse or dendritic spine density.

Glial proteins

Females had elevated GFAP relative to males (Fig. 6C; $F_{(1, 50)}$ = 10.01, p = 0.003; n=13-14/group) and there was a trend for E4FADs to have elevated GFAP relative to E3FADs (Fig. 6C; $F_{(1, 50)}$ = 3.8, p = 0.057). These effects were driven by elevated levels of GFAP in female E4FADs compared to every other group (p = 0.004 vs male E3FAD; p = 0.06 vs female E3FAD; and p = 0.008 vs male E4FAD) and suggest a potential increase in dorsal hippocampal astrocytes or astrocytic activation among female E4FADs. In contrast to GFAP, there were no effects of sex or genotype, nor interactions, in levels of Iba1 (Table 2; n=13-14/group), indicating a lack of sex and *APOE* genotype effects on microglial protein expression in the dorsal hippocampus.

Membrane-associated proteins

ERα interacts with mGluR1 at the cell membrane to increase phosphorylation of the 42-kDa isoform of ERK (p42 ERK) and cyclic-AMP response element binding protein (pCREB; Boulware et al., 2005), and increased p42 ERK phosphorylation is necessary for ERα activation to enhance memory in the OR and OP tasks (Boulware et al., 2013).

Here, E3FADs exhibited lower ERα levels than E4FADs (Fig. 6D; $F_{(1, 47)} = 5.88$, $p = 0.02$; n=11-14/group), an effect driven by male E3FADs, whose ERα levels were lower than E4FADs of both sexes ($F_{(1,15)} = 5.67$, $p < 0.03$; $p = 0.03$ vs male E4FAD; $p = 0.04$ vs female E4FAD). No effects of sex or *APOE* genotype were observed for ERβ protein expression (Table 2, n = 13-14/group), indicating that the effect of *APOE* genotype on nuclear estrogen receptor protein expression was specific to ERα. In addition, caveolin-1 protein levels were not affected by sex or genotype (Table 2; n=11-16/group).

Cell-signaling proteins

Males expressed higher pCREB levels than females (Fig. 4E; $F_{(1, 45)} = 4.61$, $p = 0.04$; n=10-15/group). In contrast, no effects of sex or genotype, nor any interactions, were observed for p42 ERK, pPI3K, p4EBP, pcofilin, p46 JNK, or p54 JNK (Table 2; n=9-15/group), suggesting that these phospho proteins are not upregulated in the dorsal hippocampus of EFAD mice 5m after learning.

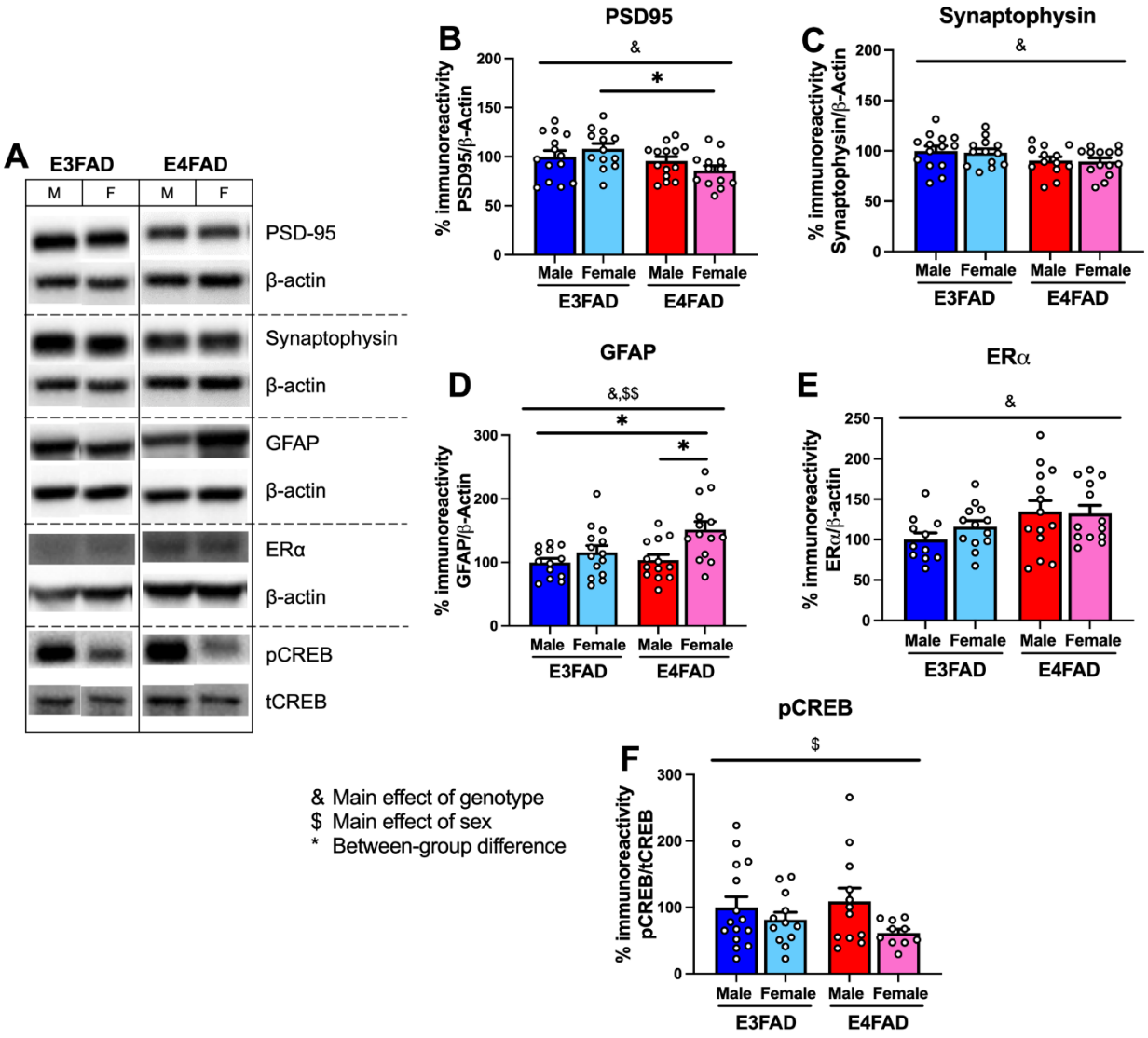


Fig. 6. E4FAD mice, particularly E4FAD females, displayed altered levels of several dorsal hippocampal proteins. (A) Representative blots illustrate relative group differences in protein expression. (B,C) Synaptic proteins PSD95 and synaptophysin were decreased in E4FADs relative to E3FADs ($^{\&}p < 0.05$). (D) GFAP protein expression was increased in female EFADs relative to male EFADs ($^{\$}p < 0.05$, main effect of sex) and tended to be higher in E4FADs than in E3FADs ($^{\&}p = 0.057$). (E) ERα levels were significantly higher in E4FADs than in E3FADs ($^{\&}p < 0.05$). (F) Levels of pCREB were significantly higher in male EFADs relative to female EFADs ($^{\$}p < 0.05$). $^{*}p < 0.05$ = between-group differences, $^{\&}p < 0.05$ = main effect of genotype, $^{\$}p < 0.05$ = main effect of sex. Bars represent the mean ± SEM.

Table 2. Proteins in dorsal hippocampus unaffected by sex or *APOE* genotype

Protein*	E3FAD		E4FAD	
	Male	Female	Male	Female
Iba1	100.0 ± 7.52	93.84 ± 5.90	104.9 ± 7.67	113.4 ± 9.48
Caveolin-1	100.0 ± 5.09	100.6 ± 9.93	110.1 ± 9.39	108.3 ± 12.5
ERβ	100.0 ± 5.41	98.54 ± 5.72	102.4 ± 5.1	106.8 ± 4.35
p42 ERK	100.0 ± 7.48	115.85 ± 10.65	94.31 ± 5.68	98.29 ± 9.13
pPI3K	100.0 ± 7.78	98.78 ± 5.44	91.82 ± 6.13	97.14 ± 8.47
p4EBP	100.0 ± 8.35	130.38 ± 12.25	110.15 ± 11.3	108.37 ± 13.86
pcofilin	100.0 ± 9.67	106.5 ± 16.1	114.5 ± 12.5	109.9 ± 9.86
p46 JNK	100.0 ± 2.97	101.2 ± 3.09	99.06 ± 3.15	104.2 ± 3.25
p54 JNK	100.0 ± 4.12	108.7 ± 3.85	99.93 ± 5.17	103.0 ± 3.88

*All proteins normalized to the male E3FAD group, whose values were set to 100.

Values represent mean % immunoreactivity (± SEM) for the target protein divided by b-actin (Iba-1, Caveolin-1, ERβ) or unphosphorylated total protein (all phospho proteins).

Dendritic analyses

We next examined apical and basal dendritic spine density in hippocampal area CA1 and in the prelimbic/infralimbic area of the mPFC to assess whether sex and *APOE* genotype influence dendritic spine anatomy. Neither sex nor genotype affected the density of apical spines in CA1 or mPFC (Table 3). Sex also did not affect basal spines in either brain region. Effects of genotype on basal spines are detailed below.

CA1

Total CA1 basal spines varied significantly by genotype ($F_{(1, 23)} = 8.75$, $p = 0.007$; n=6-7/group), such that E4FADs had reduced total basal spine density compared to E3FADs (Fig. 7A, B). Differences in total basal spine density were driven by alterations in mushroom spines (Fig. 7C; $F_{(1, 23)} = 3.96$, $p = 0.059$) and stubby spines (Fig. 7D; $F_{(1, 23)} = 8.75$, $p = 0.007$). In both cases, E4FADs exhibited lower density than E3FADs. Stubby spine density was lower in male E4FADs than in female E3FADs ($p = 0.05$). Neither sex nor genotype affected CA1 basal thin spine density (Table 3). Combined, these data indicate that *APOE4* genotype contributes to a selective reduction in basilar spine density in the dorsal hippocampus relative to *APOE3* genotype. That mushroom and stubby spines were specifically affected suggests that E3FADs have more mature and intermediate spines on basilar dendrites relative to E4FADs.

Table 3. CA1 and mPFC basal thin and apical spine density measures unaffected by sex and genotype

Brain region	Spine type	E3FAD		E4FAD	
		Male	Female	Male	Female
CA1	Basal thin	3.55 ± 0.28	3.59 ± 0.33	3.70 ± 0.28	3.67 ± 0.13
	Apical total	15.5 ± 0.61	15.7 ± 1.11	15.7 ± 0.50	15.6 ± 0.38
	Apical mushroom	3.70 ± 0.40	3.37 ± 0.34	3.79 ± 0.34	3.64 ± 0.19
	Apical stubby	5.80 ± 0.50	5.71 ± 0.31	5.86 ± 0.56	5.95 ± 0.36
	Apical thin	5.89 ± 0.34	6.35 ± 0.53	5.91 ± 0.34	5.77 ± 0.30
mPFC	Basal thin	2.14 ± 0.11	2.54 ± 0.23	2.42 ± 0.22	2.50 ± 0.41
	Apical total	9.22 ± 0.31	9.07 ± 0.22	8.77 ± 0.30	8.67 ± 0.35
	Apical mushroom	3.24 ± 0.18	3.23 ± 0.34	2.99 ± 0.41	2.95 ± 0.34
	Apical stubby	2.95 ± 0.36	2.65 ± 0.19	2.51 ± 0.18	2.41 ± 0.17
	Apical thin	2.92 ± 0.17	3.15 ± 0.32	3.29 ± 0.33	3.30 ± 0.33

Values represent mean spines/10 μM ± SEM.

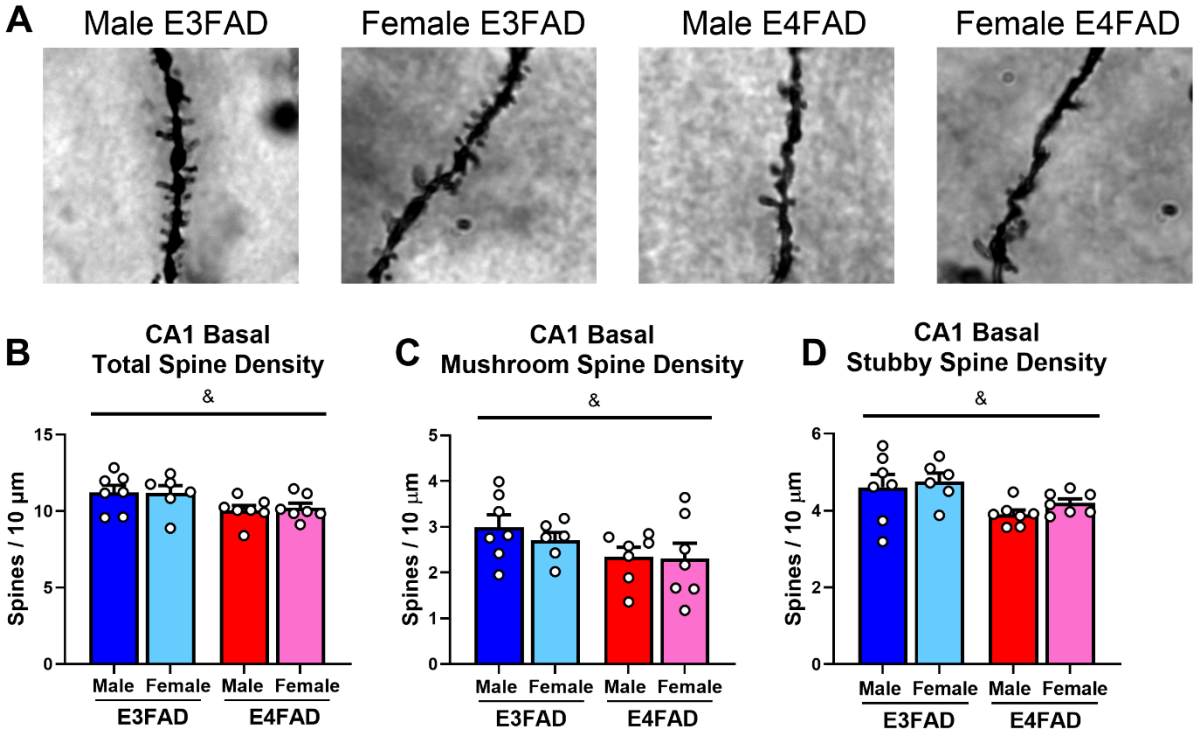


Fig. 7. E4FADs of both sexes exhibited reduced CA1 dendritic spine density. (A) Representative images at 100x of basilar dendritic segments in CA1 illustrate relative differences in spine density. (B) E4FADs exhibited lower total CA1 basal spine density relative to E3FADs ($^{\&}p < 0.05$, main effect of genotype). In particular, E4FADs exhibited reduced numbers of mushroom (C; $^{\&}p = 0.059$) and stubby (D; $^{\&}p < 0.05$) spines relative to E3FADs. Bars represent the mean ± SEM.

Sholl analysis was used to evaluate whether sex acts independently or concordantly with *APOE3* or *APOE4* genotypes to alter dendritic branching complexity within the CA1. Dendritic intersections with concentric spheres placed at successive 10 µm intervals from the cell soma were quantified. The pattern of dendritic branching was similar for all groups; dendritic intersections increased in all groups until a distance of approximately 100 µm (apical) or 60 µm (basal) and then declined (Fig. 8). Although the main effect of

distance from the soma was significant for both apical ($F_{(37,851)}$ = 240.0, p < 0.0001) and basal ($F_{(2.492, 57.31)}$ = 350.8, p < 0.0001) dendritic intersections, sex and *APOE* genotype did not influence morphological complexity of dorsal hippocampal CA1 pyramidal neurons.

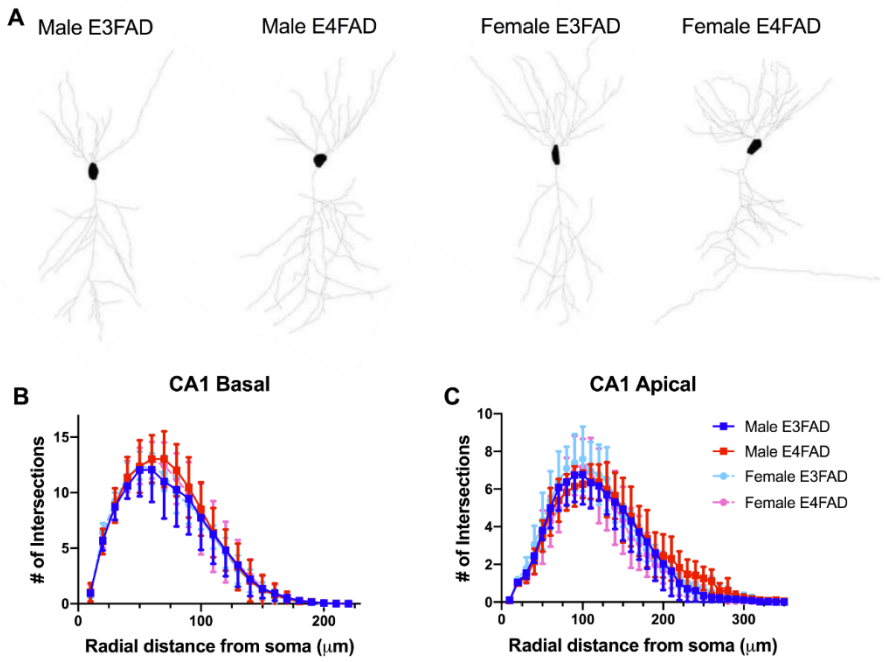


Fig. 8. Sholl analysis of CA1 dendritic branching complexity yielded no differences among the groups. (A) Representative tracings of pyramidal cells from EFAD mice. (B) No group differences in branching complexity were observed in either basal (A) or apical (C) dendrites. Mean number of intersections ± SEM are plotted.

mPFC

The effects of genotype on dendritic spine density in the mPFC were identical to those observed in CA1 (n=5-7/group). E4FADs exhibited reduced total mPFC basilar spine density compared to E3FADs (Fig. 9A,B; $F_{(1, 22)}$ = 31.92, p < 0.0001). Total basal spine density was higher

in male and female E3FADs than in E4FADs of both sexes (p = 0.0008-0.01). As with basilar CA1 spines, the genotype effect in total mPFC basal spine density was driven by decreased mushroom (Fig. 9C; $F_{(1, 22)}$ = 15.02, p = 0.0008) and stubby (Fig. 9D; $F_{(1, 22)}$ = 5.12, p = 0.03) spine density in E4FADs relative to E3FADs. Relative to E4FADs of both sexes, basal mushroom spine density was higher in male and female E3FADs (p = 0.035-0.04).

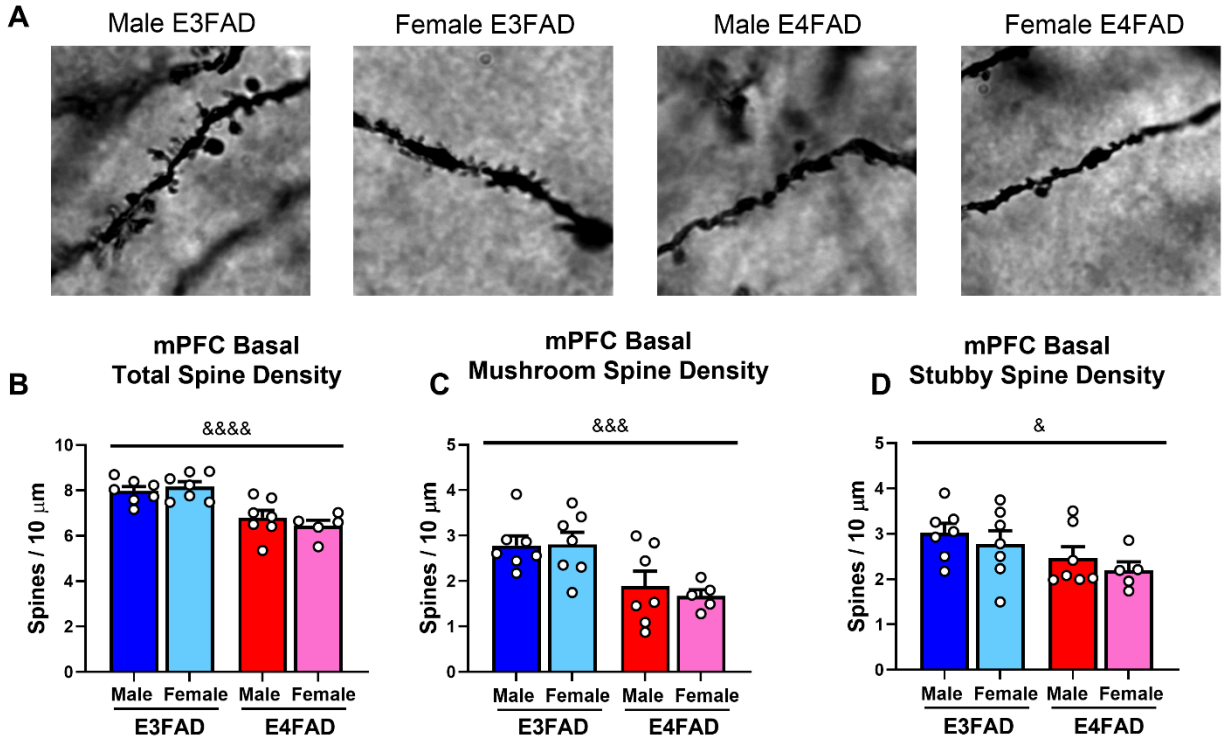


Fig. 9 E4FADs of both sexes exhibited reduced mPFC dendritic spine density. (A) Representative images at 100x of basilar dendritic segments in mPFC illustrate relative differences in spine density. (B) E4FADs exhibited lower total mPFC basal spine density relative to E3FADs ($^{\&}p < 0.05$, main effect of genotype). Notably, E4FADs exhibited reduced numbers of mushroom (C) and stubby (D) spines relative to E3FADs ($^{\&}p < 0.05$). Bars represent the mean ± SEM.

Discussion

The neural mechanisms underlying the increased risk of AD to women *APOE4* carriers are unclear, thus necessitating a better understanding of how sex and different *APOE* genotypes influence cognition and brain function. Here, we used an EFAD mouse model of AD to examine whether sex modulated differences between *APOE3* and *APOE4* genotypes in mnemonic function, protein expression, and dendritic morphology against the backdrop of AD-like pathology previously reported in this model (Tai et al., 2017; Youmans et al., 2012). We hypothesized that E4FAD mice (*APOE4*$^{+/+}$/5xFAD$^{+/-}$) would exhibit impaired memory, reduced hippocampal levels of synaptic, membrane, and cell-signaling proteins, increased hippocampal levels of glial proteins, and decreased dendritic spine density and branch complexity in CA1 and mPFC relative to 5xFAD mice expressing two copies of human *APOE3* (E3FAD). We further expected these changes to be most pronounced in female E4FAD mice. The findings suggest that *APOE4* genotype impaired spatial and object recognition memory consolidation in object-based tasks, reduced hippocampal synaptic markers, and decreased dendritic spine density in the CA1 and mPFC relative to *APOE3*, yet increased hippocampal levels of ERa (Fig. 7). We observed surprisingly few sex differences or sex x genotype interactions in all measured outcomes, suggesting that the known synergistic effects of *APOE4* and female sex are likely modulated by factors not measured in the present study.

Interestingly, sex differences in object-based memory were observed among E3FADs, but not E4FADs. Specifically, only male E3FAD mice remembered the identity and location of the training objects in the OR and OP tasks, suggesting impaired object recognition and spatial memory consolidation not only in E4FADs of both sexes but also

in E3FAD females. However, the sex differences in memory observed within E3FADs were not reflected in any measure of neuronal structure or function, so the underlying mechanisms remain unclear. This preserved brain function in E3FAD females may have allowed them to find the platform in the MWM as well as E3FAD males, as E3FADs of both sexes improved similarly across sessions in this task. Curiously, E3FADs of both sexes had lower dorsal hippocampal Erα expression than E4FADs, suggesting either a potential role for high ERα levels in *APOE4*-induced memory impairments, or a decrease in ERα in E3FADs that serves to maintain memory. Levels of synaptic proteins and the density of mature and developing basal dendritic spines in the CA1 and mPFC were reduced in E4FAD mice of both sexes relative to male and female E3FAD mice, suggesting a detrimental effect of *APOE4* genotype, but not sex, on spine synapses and synaptic plasticity. However, sex did affect levels of the astrocytic protein GFAP and the transcription factor pCREB, with females expressing more GFAP and less pCREB. Interestingly, female E4FADs expressed substantially higher levels of GFAP than E4FAD males. These findings suggest increased astrogliosis in E4FAD females. Together, the results indicate limited effects of sex on memory in EFAD mice, with no sex differences observed among E4FADs in either task, and a sex difference (favoring males) only evident among E3FADs in the two object tasks. These sex differences were not reflected in any neurobiological measure, although sex differences unrelated to memory were observed in GFAP and pCREB among E4FAD mice.

In previous work with C57BL/6 mice, ovariectomized females and gonadally-intact or castrated males show intact memory in the OR and OP tasks when tested 24 or 4 h later, respectively (Fortress et al., 2013; Kim et al., 2019; Koss et al., 2018; Taxier et al.,

2019). Thus, these are delays at which wild-type mice can remember the identities and locations of the training objects. Here, only male E3FAD mice exhibited intact memory in both tasks. The superior object memory of E3FAD males compared to E4FADs of both sexes is consistent with previous reports of reduced pathology in E3FAD males relative to other EFADs (Youmans et al., 2012) and literature suggesting that *APOE3* is less detrimental to memory than *APOE4* in both animal models and human AD patients (Beydoun et al., 2012; Liu et al., 2015). In support, the impaired memory exhibited by male and female E4FADs, as well as female E3FADs, is consistent with previous work showing spatial and object memory impairments in rodent models of AD (Ardiles et al., 2012; Ashe, 2001). Moreover, other AD mouse model studies report exacerbated memory impairment in females relative to males (Schmid et al., 2019; Yang et al., 2018; Yue et al., 2011). Thus, our data showing that female EFADs of both genotypes could not remember the identity and location of previously seen objects are consistent with these findings. Although we expected that all groups but female E4FADs would display intact memory, AD pathology may have been sufficient by 6M to impair memory in male and female E4FADs and in female E3FADs. E4FAD mice exhibit significant AD pathology and behavioral deficits as early as 4M, especially relative to E2FAD mice (Liu et al., 2015), so had we tested the mice at an earlier age, we may have captured sex differences in memory within the E4FADs.

In contrast to the object tasks, all mice could learn to find the platform in the MWM task. This is somewhat surprising, as swimming through a large water tank to find a hidden platform seems inherently more complex than exploring objects in an open field. Nevertheless, the lack of impairment among E4FADs is consistent with a previous

comparison of 6 month-old female E3FAD and E4FAD mice showing minimal MWM deficits in E4FADs relative to E3FADs (Liu et al., 2015). MWM training is much more extensive than the object tasks and the number and length of training trials per day, in addition to the robustness of room cues during water maze testing, may have greatly aided spatial memory formation in the MWM among mice from all groups. A more challenging training protocol (e.g., fewer trials or fewer cues) may have captured between-group differences.

Because synaptic dysfunction is a key feature in AD (Masliah et al., 2006; Reddy et al., 2005), we investigated whether sex and *APOE* genotype influence expression of synaptic proteins within the dorsal hippocampus of EFAD mice. Our data showing a reduction in PSD95 and synaptophysin levels in E4FADs relative to E3FADs, particularly among females, is consistent with previous work showing reduced hippocampal PSD95 levels in 4 month-old female E4FAD mice relative to E3FAD females (Liu et al., 2015). Our findings are also consistent with clinical data demonstrating that expression of synaptophysin is markedly decreased in AD patients compared to healthy controls (Heinonen et al., 1995), and in *APOE4*-expressing individuals relative to *APOE3* carriers (Love et al., 2006).

The lower levels of hippocampal synaptic proteins observed in E4FADs relative to E3FADs are consistent with the reduced CA1 and mPFC basal dendritic spine density also evident in E4FADs. Previous findings in *APOE*-TR mice indicate a detrimental effect of *APOE4* on cortical, but not hippocampal, spine density across aging (Dumanis et al., 2009), although others using this model have reported an adverse effect of *APOE4* on spine density in the hippocampus (Ji et al., 2003). The decreased mushroom spine

density observed in E4FADs suggests that E3FADs have more mature spines than E4FADs, which is consistent with the overall negative effect of *APOE4* genotype on memory processes. Our finding of more stubby spines in E3FADs than E4FADs may reflect ongoing cytoskeletal reorganization in dendritic spines. Previous work in a different mouse model of AD suggests that stubby spines may be more dynamic than other spine types (Spires-Jones et al., 2007). Because spine remodeling is a continual process (Kasai et al., 2010), and our own data present only a snapshot of this process at a single timepoint, we speculate that elevated stubby spine density in E3FADs reflects greater spine plasticity, motility, recycling, and/or remodeling relative to E4FADs.

Moreover, the negative impact of *APOE4* genotype on basal, but not apical, spine density, in the current study suggests the intriguing possibility that *APOE4* genotype does not uniformly modulate spine density, but rather influences distinct sites of synaptic input within both the mPFC and the dorsal hippocampus. Given reports that basal dendrites in mPFC receive input from thalamic projections, and that communication between the dorsal hippocampus and mPFC may be facilitated via the nucleus reuniens of the thalamus (Hoover & Vertes, 2007), future work should interrogate whether changes in basal dendritic spine density reflect compromised neurocircuitry between the dorsal hippocampus and mPFC in EFAD mice.

In addition to neuronal morphology, we examined expression of glial proteins because both astrocytic and microglial reactivity is closely linked to AD pathology (Meda et al., 2001). Although neither sex nor *APOE* genotype affected microglial Iba-1 expression in the dorsal hippocampus, astrocytic protein GFAP was elevated in female EFADs relative to male EFADs, and in E4FADs relative to E3FADs. Previous work

showed striking differences in astrocytic reactivity between male E4FAD and E3FADs (Tai et al., 2017), with E4FADs having elevated GFAP expression relative to E3FADs. The present genotype effect differs in that it is primarily driven by elevated GFAP in female E4FADs. The sex-specific effect on levels of GFAP, as well as the particularly high GFAP levels in female E4FADs, suggests that both female sex and *APOE4* genotype interact to increase susceptibility to astrogliosis.

In our examination of cell-signaling proteins that are rapidly activated in response to learning, we found that dorsal hippocampal pCREB levels were increased in male EFADs relative to female EFADs. In the hippocampus of AD patients, CREB-mediated transcriptional outcomes are dysregulated (Satoh et al., 2009), and pCREB is reduced relative to age-matched controls (Yamamoto-Sasaki et al., 1999), suggesting that aberrant hippocampal CREB signaling is a feature of AD. Although sex differences in CREB-associated signaling remain poorly characterized in rodent models of normal or pathological aging, previous work with CREB-deficient aging mice showed that aberrant CREB signaling resulted in reduced spatial memory in females compared to males (Hebda-Bauer et al., 2007). In addition, female 3xTg mice exhibited lower pCREB expression relative to male 3xTg mice (Yang et al., 2018). These data are in line with our own, suggesting that reduced pCREB is particularly exacerbated in female E4FADs relative to male E4FADs. We were surprised to find no additional genotype or sex-specific effects on levels of other cell-signaling proteins that are critical for memory processes; however, one training session with novel objects prior to tissue collection may not have been sufficient to drive learning-induced changes in these pathways. Future work should

examine multiple time points after a robust learning experience to more definitively determine whether cell-signaling activity is compromised by *APOE4* genotype.

Our finding that dorsal hippocampal ERα levels are increased in E4FADs relative to E3FADs is consistent with previous work showing that ERα levels are increased in the AD brain (Ishunina et al., 2003; Ishunina & Swaab, 2003). Caveolin-1 plays a key role in associating mGluRs with ERα at the cell membrane (Boulware et al., 2013; Razandi et al., 2002), although caveolin-1 protein levels were unaffected by sex or genotype in the present study. Therefore, the increase in ERα levels seen among E4FADs relative to E3FADs suggests that *APOE4* may promote an increase in the cytosolic, rather than membrane, localization of this receptor. Others have posited that estrogens may interact with ERα to increase levels of apolipoprotein E (apoE), particularly in *APOE4* carriers, in a manner that would make such individuals more vulnerable to disease progression (J. M. Wang et al., 2006). Although our data lend correlative support to this hypothesis, any differential effect of *APOE* on estrogen therapy remains unclear. Notably, our data indicate that ERβ expression was unaffected by *APOE* genotype, thus implicating ERα as a more promising target for estrogen therapy for *APOE4* carriers.

Collectively, the results from the present study of 6 month-old EFAD mice suggests a substantial influence of *APOE4* genotype, but not sex, on measures of hippocampus-dependent memory, and hippocampal spine density, and protein expression. Of particular note, *APOE4* genotype had detrimental effects on object memory, CA1 spine density, and dorsal hippocampal levels of synaptic proteins and ERα. E4FAD females additionally exhibited aberrant GFAP levels and CREB phosphorylation. Future studies are critical to determining whether other aspects of neural function can account for the sex and *APOE*

genotype interactions observed in humans that increase *APOE4*+ women's vulnerability to AD. Knowing when *APOE4* and sex exert detrimental effects is a key knowledge gap, and the present study is limited by the examination of these variables at a single time point in EFAD mice. Therefore, next steps should include examining whether *APOE4* and sex negatively impact synaptic integrity and memory at an earlier age, and whether the possibility exists to reverse or mitigate the course of neurodegeneration modulated by *APOE4* genotype and sex. Pinpointing the time at which memory deficits emerge may have therapeutic potential in resolving memory decrements and associated neuropathology (Lanfranco et al., 2020; Tai et al., 2014).

CHAPTER THREE. Defining the impact of APOE genotype and ovariectomy on anxiety-like behavior in EFAD mice

The primary objective of this aim was to determine whether *APOE* genotype and gonadal status (i.e. intact or ovariectomized) act independently or together to modulate anxiety-like behavior. Interestingly, previous evaluations of anxiety-like behavior in rodent models of AD have yielded inconsistent and contradictory results. In some cases, transgenics including APP/PS1 and 5xFAD mice, exhibited decreased or equivalent anxiety-like behavior in the open field or elevated plus maze relative to wild-type controls (Arendash et al., 2001; Jawhar et al., 2012; Radde et al., 2006). By contrast, increased anxiety-like behavior has also been reported, often in the same tasks or animal model that reportedly exhibited decreased anxiety-like behavior in previous studies (Flanigan et al., 2014; Lippi et al., 2018; Sterniczuk et al., 2010). Discordant findings in investigations of anxiety-like behaviors underscore the need for additional attention to be paid to modeling anxiety-like behaviors in rodent models of AD and determining how risk factors like *APOE* genotype, sex, and ovarian hormone loss influence these behaviors within these models.

Consistent with the human literature, male and female mice expressing *APOE4* exhibit increased anxiety-like behavior relative to *APOE3* carriers (Robertson et al., 2005; Siegel et al., 2012). However, whether this between-genotype difference persists in model in which mice also develop AD-like pathology remains an important question. In addition, although female sex increases risk for anxiety and for AD in humans, few existing studies evaluate sex differences in anxiety-like behavior in mouse models of AD. Unfortunately, many studies that include both males and females do not segregate data by sex, which

could inadvertently occlude observation of sex differences in anxiety-like behaviors in these models. Given the differential contributions of sex, as well as *APOE3* and *APOE4* genotypes, to anxiety in human patients (Michels et al., 2012; Tao et al., 2018), examining how these factors contribute to anxiety-like behavior against a background of AD pathology is a crucial step towards targeting anxiety-like symptomology in disease treatments. Our working hypothesis was that *APOE4* genotype and ovariectomy would increase anxiety-like behavior in EFAD mice.

Materials and methods

Subjects

Female EFAD mice homozygous for *APOE3* or *APOE4* underwent the same breeding process and aging process as in aim 1. Mice were housed in groups of 3-5 per cage (at both UIC and UWM for Experiment 1a, designed to test impact of *APOE* genotype on anxiety-like behavior) or singly housed (Experiment 1b, designed to test the combined impact of *APOE* genotype and ovariectomy on anxiety-like behavior) and maintained on a 12 hr light/dark cycle with *ad libitum* access to food and water for the duration of the study. Mice were handled for 30 s/day for three days prior to behavior. Protocols and procedures followed the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the UIC Animal Care Committee and UWM Institutional Animal Care and Use Committee.

Surgery

Mice in Experiment 1a did not undergo surgical procedures. Two weeks prior to behavioral testing, female E3FAD and E4FAD mice in Experiment 1b underwent bilateral ovariectomy to eliminate ovarian hormones, and were also implanted with bilateral stainless steel cannulae into the dorsal hippocampus as described previously (Lewis et al., 2008) for subsequent experiments. During the procedure, mice were anesthetized with isofluorane (5% for induction, 2% for maintenance) in 100% oxygen. For analgesia, mice received carprofen MediGel 1 day before and 1 day after surgery, and received a subcutaneous injection of 5 mg/kg Rimadyl at the start of the surgical procedure.

Open Field

Data were collected during a 5-minute trial, during which mice explored a novel empty square arena (60 cm x 60 cm x 47 cm). The arena was illuminated by standing lights to approximately 75 lux. A luxometer was used before training to verify that the four corners of the OF were evenly illuminated and within 5 lux of each other. OF was used to measure general locomotor activity and time spent in the perimeter as an indicator of anxiety-like behavior (Prut and Belzung, 2003). The arena was divided into 16 squares via an automated grid system using ANYmaze software (San Diego Instruments), which was also used to record data. Time in the center squares of the arena, distance travelled, and speed were recorded. More time in the center was indicative of low anxiety levels, and higher values for distance and speed indicated increased activity (Bailey & Crawley, 2009).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 8 software. For Experiment 1a, differences were assessed using two-way ANOVAs with sex and genotype as between-subject variables. For Experiment 1b, differences were assessed using two-way ANOVAs with genotype and gonadal status as between-subject variables. Because *APOE4* genotype and female sex are associated with greater anxiety in humans (Michels et al., 2012; Tao et al., 2018), we hypothesized that female E4FADs would exhibit the most anxiety-like behavior followed by female E3FADs and male E4FADs, and then male E3FADs. Due to these specific predictions, significant main effects were followed by planned Tukey's *post hoc* comparisons. Statistical significance was set at $p < 0.05$ for all statistical tests. Trends were determined by $p < 0.10$. Effect sizes were calculated using η^2.

Results

APOE4 genotype increases anxiety-like behavior in the open field relative to APOE3 genotype

Given the prevalence of anxiety in AD patients (Ferretti et al., 2001; Porter et al., 2003), particularly *APOE4* carriers (Michels et al., 2012), anxiety-like behaviors were measured in male and female E3FAD (n = 16, male E3FAD; n = 16, female E3FAD) and E4FAD mice (n = 13, male E4FAD; n = 14, female E4FAD) using the open field (OF).

E4FADs, regardless of sex, spent significantly less time in the center of the OF than E3FADs (Fig. 10B; genotype effect: $F_{(1, 55)} = 6.89$, $p = 0.01$, $\eta^2 = 0.11$). This effect

was prominent in E4FAD females, such that female E4FADs spent significantly less time in the center than female E3FADs (p = 0.04). The main effect of sex and genotype x sex interaction were not significant.

Females traveled further than males (Fig. 10C; $F_{(1, 53)}$ = 8.0 p = 0.007, η^2 = 0.13). Female E4FADs traveled the furthest, moving longer distances than male E3FADs (p = 0.02). Average speed was also influenced by sex, such that females traveled faster than males (Fig. 10D; $F_{(1, 53)}$ = 8.07, p = 0.006, η^2 = 0.13). As with distance traveled, female E4FADs traveled significantly faster than male E3FADs (p = 0.02). The main effects of genotype and genotype by sex interactions were not significant for either measure.

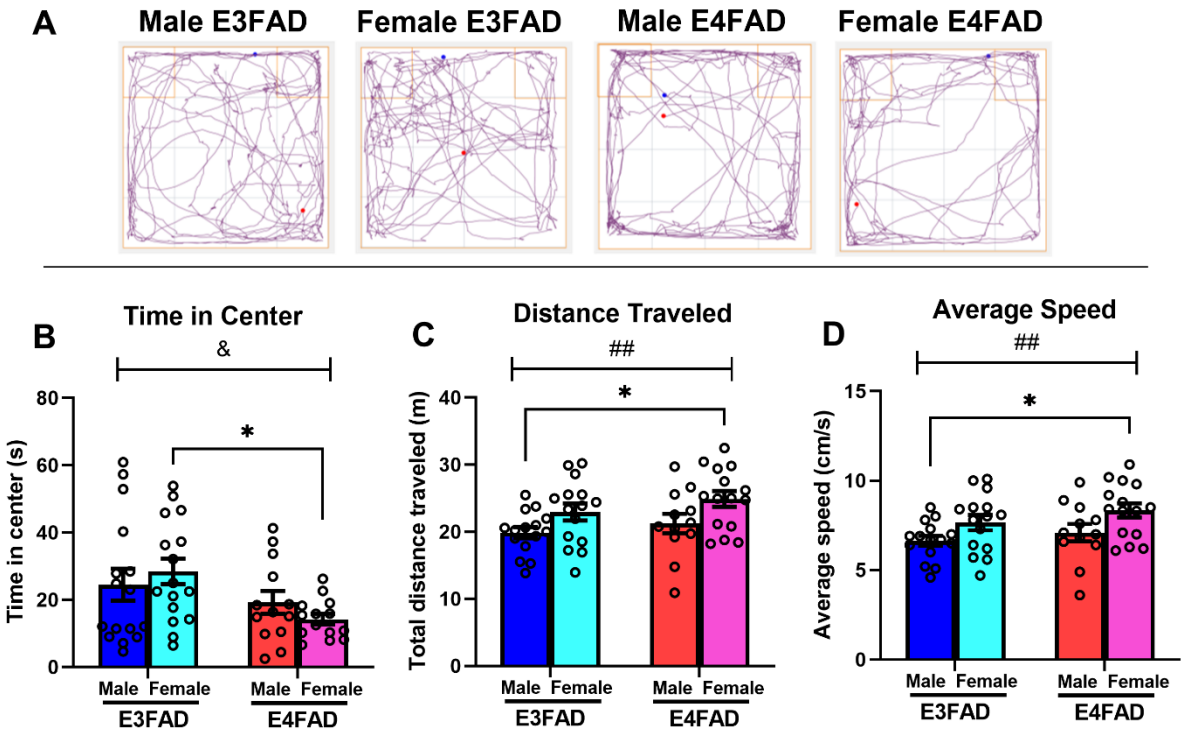


Fig 10. E4FADs exhibit more anxiety-like behavior in the open field than E3FADs. (A) Track plots illustrate representative patterns of movement in the open field. (B) E4FAD females spent less time in the center relative to E3FADs ($^{\&}p < 0.05$, main effect of genotype; $^{*}p < 0.05$, female E4FADs vs female E3FADs). (C,D) Female E4FADs traveled further ($^{\#\#}p < 0.01$, main effect of sex; $^{*}p < 0.05$, female E4FAD vs male E3FAD) and faster on average ($^{\#\#}p < 0.01$, main effect of sex; $^{*}p < 0.05$, female E4FAD vs male E3FAD) than male E3FADs.

Ovariectomy increases anxiety-like behavior in the open field in E3FADs

Because oophorectomy before menopause increases AD risk (Rocca et al., 2007), and estrogens are anxiolytic (Walf & Frye, 2006), we sought to determine whether loss of ovarian hormones facilitates increased anxiety-like behavior in the OF in female EFADs. Here, we hypothesized that female E4FADs would spend significantly less time in the center of the OF than every other group, and that OVX would exacerbate this effect. Thus, we tested whether bilateral ovariectomy resulted in decreased time spent in the center of the OF in the EFAD model by comparing anxiety indices of the intact female E3FAD and E4FADs from Experiment 1a to OVXed female E3FAD (n = 32) and E4FADs (n=31).

OVXed female EFADs spent significantly less time in the center of the OF relative to intact female EFADs (Fig. 11B; gonadal status effect: $F_{(1,87)} = 16.6$, $p < 0.0001$, $\eta^2 = 0.15$), and E4FADs spent less time in the center relative to E3FADs (Fig. 11B; genotype effect: $F_{(1,87)} = 4.14$, $p = 0.045$, $\eta^2 = 0.04$). These effects and their interaction (Fig. 11B; $F_{(1,87)} = 6.98$ $p = 0.01$, $\eta^2 = 0.06$) were driven by intact female E3FADs spending significantly more time in the center than every other group ($p = 0.006$ vs OVXed E3FADs; $p = 0.0005$ vs intact E4FADs; $p = 0.0002$ vs OVXed E4FADs). Importantly, no differences were observed between OVXed E3FAD and E4FAD females, or intact and OVXed E4FADs, suggesting that OVX induces an E4FAD-like OF phenotype in female E3FADs.

For distance traveled and average speed, two-way ANOVAs yielded no significant main effects or interactions, suggesting that all groups traveled similar distances at similar speeds.

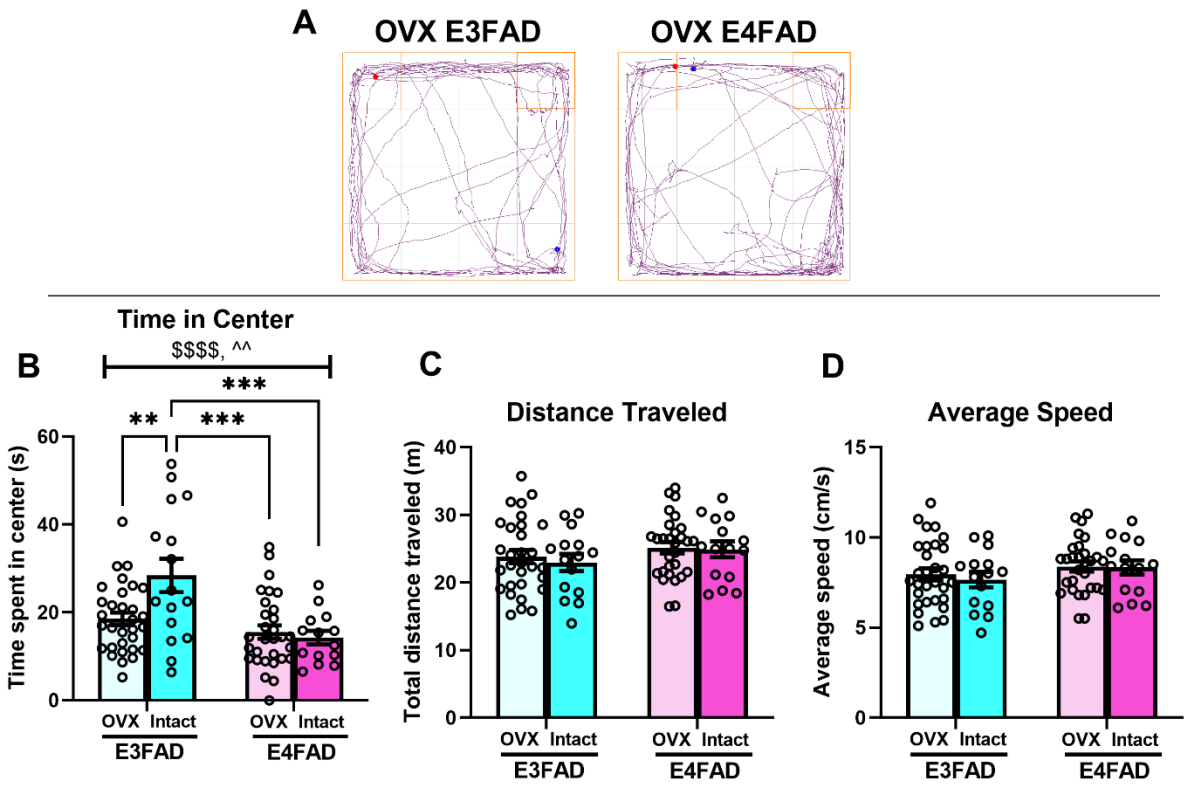


Fig. 11. Ovariectomy induces an *APOE4*-like anxiety phenotype in E3FAD females. (A) Track plots illustrate representative patterns of movement in ovariectomized EFADs in the open field. (B) E3FAD females spent significantly more time in the center than E4FAD females ($^{\&}p < 0.05$, main effect of genotype), and intact E3FADs spent significantly more time in the center than OVXed EFADs ($^{\$\$\$}p < 0.0001$, main effect of OVX). Intact E3FADs spent significantly more time in the center relative to every other group ($^{\wedge\wedge}p < 0.01$, significant interaction; $^{**}p < 0.01$ vs OVXed E3FADs, $^{***}p < 0.001$ vs intact and OVXed E4FADs). (C,D) Distance traveled and average speed did not differ among female EFADs regardless of genotype or gonadal status.

Discussion

Given the prevalence of anxiety in AD patients (Ferretti et al., 2001), understanding whether genetic or sex-specific factors contribute to anxiety-like symptoms is crucial for development of therapeutics. The present study tested whether *APOE4* genotype, female sex, and ovarian hormones contribute to increased anxiety-like behavior in a mouse model of AD designed to reproduce *APOE*-associated disease risk against a background of AD-like pathology. We hypothesized that the *APOE4* genotype in this model would be associated with decreased time spent in the center of the OF relative to the *APOE3* genotype, and that OVX would be anxiogenic for OF behaviors. Results indicate that *APOE4* genotype was anxiogenic in the OF, as was ovarian hormone loss in female *APOE3* homozygotes. These data provide new insights into factors that may contribute to anxiety in women with AD, and suggest that hormone therapy may help alleviate anxiety in some female patients.

In male and female mice expressing human *APOE*, *APOE4* carriers exhibit more anxiety-like behavior than *APOE3* carriers (Robertson et al., 2005; Siegel et al., 2012). Data from human patients with probable AD mirror findings from animal models, in that *APOE4* is anxiogenic relative to *APOE3* (Robertson et al., 2005). These findings are consistent with our data demonstrating that E4FADs spent less time in the center of the OF compared to E3FADs, although this effect appears to be driven largely by female E4FADs. Others have found the *APOE4* genotype to be anxiogenic in males in the elevated plus maze (Robertson et al., 2005). However, our results suggest minimal differences in OF behavior between male E3FAD and E4FADs, underscoring the need to use multiple behavioral tasks to provide a more complete understanding of how different

APOE genotypes influence anxiety-like behaviors. Although the sex x genotype interaction was not significant, the markedly reduced center time for female E4FADs relative to E3FADs suggests that female sex may exacerbate anxiety-like behavior in *APOE4* carriers.

We also found that female EFADs were more active than male EFADs, regardless of genotype, which is consistent with reports that female rodents are more active than males (Beatty, 1979; Rosenfeld, 2017). Although female E4FADs were the most active, they also spent the least amount of time in the center of the OF, suggesting that anxiety about being in a novel environment may have resulted in rapid circling of the periphery in an attempt to escape.

Our results demonstrating that OVXed E3FADs were more anxious than intact E3FADs, and similarly anxious to E4FAD females, are consistent with data demonstrating that OVX increases anxiety-like behavior (de Chaves et al., 2009; Schoenrock et al., 2016). However, we saw no decrease in time spent in the center of the OF in OVXed E4FADs relative to intact E4FADs. This similarity may be attributed to the detrimental impact of *APOE4*, such that the already low amount of time spent in the center of the OF in E4FAD females prevented OVX from further decreasing this measure. Given that intact female E3FADs spent more time in the center of the OF than OVXed female E3FADs, our data suggest a neuroprotective role for ovarian estrogens against anxiety-like behaviors. If validated in future studies with other anxiety-like behaviors, then these findings may suggest that both ovarian status and *APOE* genotype should be taken into careful consideration when weighing treatment options for AD patients.

Future work should also examine whether estrogen replacement is anxiolytic in E3FADs and E4FADs, and in *APOE3* and *APOE4* human carriers. Here we employed only one method of assessing anxiety-like behaviors, and examining additional indices of anxiety would provide a more complete understanding of contexts under which *APOE4* and hormone loss are anxiogenic. Two important potential confounds must be considered in future experiments examining effects of OVX on anxiety-like behaviors in EFAD mice. First, mice from Experiment 1b underwent surgical procedures, whereas mice from Experiment 1a did not. However, two weeks elapsed between surgery and behavioral testing, which allowed for full recovery prior to OF and should have mitigated any lasting effects from surgery. Nevertheless, sham OVX and intracranial surgeries would serve as important controls in future work to support a causative role of OVX and rule out any detrimental effects of cannulation surgery on brain structures involved in modulating anxiety. Second, mice from Experiment 1a were group housed, whereas mice from Experiment 1b were singly housed. It is possible that group housing protected against anxiety-like behaviors in E3FADs, and that the anxiety-like phenotype present in OVXed E3FADs cannot be fully attributed to OVX. However, because mice were only singly-housed for one week prior to testing, chronic or long-lasting effects of single housing, which is known to increase anxiety-like behaviors, is unlikely to have affected OF exploration in the present experiment. Nevertheless, disentangling the effects of single versus group housing in future work would strengthen the conclusion that OVX is responsible for an increased anxiety-like phenotype in E3FADs.

Given results showing a benefit for the potent estrogen 17β-estradiol (E_2) on cognition and anxiety-like behaviors, E_2 may also be therapeutic for anxiety in AD patients

(Walf & Frye, 2006). Furthermore, researchers should explore whether timing of administration following gonadectomy impacts the ability of E_2 to decrease anxiety-like behavior, given the critical window for estrogen replacement therapy for other cognitive behaviors. However, whether estrogen replacement therapy may resolve behavioral symptomology of AD in *APOE4* carriers remains controversial and deserves further study.

CHAPTER FOUR. Determining whether 17-β estradiol can facilitate memory in female E3FAD, E3/4FAD, and E4FAD mice

The primary objectives of this study were to determine: 1) whether E_2 was of mnemonic benefit to ovariectomized EFAD females, and 2) whether changes in dendritic spine density and protein expression underly the beneficial effects of E_2 in E3FAD and E3/4FAD, but not E4FAD females. Because the drop in circulating ovarian hormones is associated with cognitive disruptions in women (Mitchell & Woods, 2011; Weber et al., 2014), whether exogenous estrogen replacement is of mnemonic benefit in both normal and pathological aging has been a long-standing and sometimes controversial question within the field of cognitive neuroendocrinology (McCarrey & Resnick, 2015). Our own data show that intracranial administration of estrogens can extend the delay at which ovariectomized female mice can remember a previously seen object or location (Fortress et al., 2013; Kim et al., 2016; Taxier et al., 2019). Until the current project, however, we had not explored whether intrahippocampal E_2 was beneficial in a mouse model of AD.

E_2 reduces risk for cognitive impairment in *APOE4*-negative, but not *APOE4* positive female patients (Yaffe et al., 2000). Limited available data suggest that E_2 may exert similar effects in animal models of aging or disease. For example, in ovariectomized macaques, genes upregulated by exogenous estrogen treatment overlap with genes that are downregulated in brains from human AD patients (Ratnakumar et al., 2019). In the 3xTg-AD mouse model, ovariectomy resulted in cognitive deficits and increased accumulation of amyloid beta, both of which were prevented by treatment with estrogens (Carroll et al., 2007). Furthermore, the benefits of E_2 in the 3xTg model seem to be dependent upon treatment initiation during early middle age (Christensen et al., 2020),

supporting existing evidence from human patients that administration of hormone therapy must occur during a critical window of time within the onset of menopause or reproductive aging (Maki, 2013).

In the EFAD model, 3-month old OVXed E2FADs and E3FADs exhibited lower levels of extracellular amyloid and intracellular amyloid deposition in the hippocampus and cortex following chronic treatment with E_2 (Kunzler et al., 2014). Conversely, 3-month old OVXed E4FADs treated chronically over the course of 3 months with E_2 exhibited increased extracellular amyloid and intracellular amyloid deposition (Kunzler et al., 2014). These data implicate *APOE2* and *APOE3* carriers, but not *APOE4* carriers as potential beneficiaries of E_2 treatment. In fact, *APOE4* status may render individuals susceptible to deleterious effects of E_2 treatment.

Although promising evidence exists for a favorable modulatory effect of E_2 in E2FAD and E3FAD, but not E4FAD female mice on AD-like pathology (Kunzler et al., 2014), intermediary factors contributing to the beneficial effects of E_2 in E2FAD and E3FADs, and harmful effects of E_2 in E4FADs, remain unidentified. Moreover, whether a single copy of *APOE4*, rather than *APOE4* homozygosity, renders EFADs less susceptible to E_2 treatment, remains unknown. *APOE4* homozygosity is relatively uncommon in human populations, but even a single copy of *APOE4* can magnify AD risk (Farrer et al., 1997). Whether *APOE4* is similarly harmful for memory consolidation and its associated molecular processes in EFAD heterozygotes represents a key knowledge gap.

There is a paucity of data linking molecular mechanisms to the potential mnemonic benefit of E_2 in AD models that account for *APOE* status. Therefore, the present study was designed to investigate whether E_2 promotes memory consolidation in mice that are designed to recapitulate *APOE*-associated disease risk against a background of AD-like pathology. Our working hypothesis was that E_2 would enhance memory in E3FAD and E3/4FAD, but not E4FAD mice, and that E_2 would exert its effects via rapid phosphorylation of extracellular signal-regulated kinase and other kinases that interface with the mitogen-activated protein kinase cascade. In addition, based off of previous work from our lab (Tuscher et al., 2016), we hypothesized that any memory-enhancing effects of E_2 would be associated with an increase in CA1 basal and apical spine density, and mPFC basal spine density 2 h post-infusion.

Materials and methods

Subjects

Female EFAD 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). Breeding, transfer from UIC to UWM, and aging occurred as in Aims 1 and 2. Prior to surgery, mice were housed in groups of up to 5 per cage, and were singly-housed following cannulation and ovariectomy. Mice were maintained on a 12 hr 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.

Surgeries

Mice received a 5mg/kg dose of subcutaneous Rimadyl for pain management at the start of the surgical procedure. Mice were anesthetized with isofluorane in 100% oxygen (5% for induction, 2% for maintenance) and placed in a stereotaxic apparatus (Kopf instruments) for ovariectomy (OVX) and cannula implantation in the same surgical session (Lewis et al., 2008). 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 cannulae clogs. Mice received MediGel carprofen (ClearH20) for postsurgical analgesia and were given one week to recover prior to the start of behavioral testing.

Drugs and Infusions

Hydroxypropyl-β-cyclodextrin (HBC)-encapsulated E_2 (Sigma-Aldrich) was dissolved in 0.9% sterile saline to a concentration of 10μg/μl and was infused at a rate of 0.5μl/min for 1 min/hemisphere (Fortress et al., 2013). 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 an infusion of vehicle or E_2 immediately post-training. Two weeks following the conclusion of behavioral experiments,

mice were reinfused with the same treatment they had previously received, and tissue was taken 5 min following infusion for Western blotting or 2 hr following infusion for Golgi impregnation and spine counting.

Behavioral Tasks

Object recognition (OR) and object placement (OP) were conducted as described in Aim 1. Here, rather than returning mice to their home cages immediately following training, mice received DH infusion of veh or E_2 and were then returned to their home cage. Although vehicle-treated OVXed wild-type mice typically remember a previously-seen object or the location of a previously-seen object at a 24 hr (OR) or 4 hr (OP) delay, we previously showed in Aim 1 hat only male E3FADs, and not female E3FADs or E4FADs, remember the previously seen object or location (Taxier et al., submitted) when tested at these same delays. Thus, given that female E3FADs and E4FADs don't remember at a 24 h (OR) or 4 h (OP) delay, we tested animals in the present experiment using these same delays in order to test whether DH E2 infusion could facilitate memory consolidation.

Western Blotting

Two weeks following the conclusion of behavioral testing, a subset of mice (10-12/group) were infused with vehicle or E_2. Mice were cervically dislocated and decapitated 5 min post-infusion. Brains from each group were extracted and bilateral DH was immediately dissected and frozen at -80°C. Western blotting was conducted as

described previously (Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2013b; Kim et al., 2016; Koss et al., 2018; Taxier et al., 2019; Zhao et al., 2012). To prepare tissue samples for Western blot, tissues were resuspended 1:25 weight/volume in lysis buffer containing PMSF and a protease inhibitor cocktail (Sigma-Aldrich Corp.) and homogenized using a probe sonicator (Branson Sonifier 250). Homogenates are 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 verified using a ChemiDoc MP gel imager (Bio-Rad).

The following primary antibodies were used, and blots incubated overnight at 4°C: phospho-ERK (#9101, 1:2000), total-ERK (#9102, 1:2000), phospho-PI3K (#177366, 1:1000), total PI3K (#4257,1:1000), synaptophysin (#4329 1:1000), PSD95 (#2507; 1:1000), GFAP (#12389; 1:1000), Beta Actin (#4970, 1:1000), Cell Signaling Technology; ERα (H-184, 1:1000), Santa Cruz Biotechnology; ERβ (#PA1-310B, 1:1000), Thermo Fisher; and Iba1 (#016-20001, 1:1000), FujiFilm Wako Chemicals. The following day, all blots were incubated at room temperature with a rabbit secondary antibody (#7074, 1:5000, Cell Signaling Technology. Densitometry was performed using Image Lab software (Bio-Rad Image Lab v 5.2). Phosphorylated proteins were normalized to their respective total proteins. The remaining blots were stripped and reprobed for β-Actin (#4967, 1:1000, Cell Signaling Technology) for protein normalization. Data are expressed as average volume intensity as a percentage compared to vehicle-treated OVXed E3FAD females.

Golgi Impregnation and Spine Counting

Whole brains (n=4-9/group) were collected for Golgi impregnation two weeks following the conclusion of behavioral testing. Mice were cervically dislocated and decapitated 2 h following DH infusion of veh 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 (Tuscher et al., 2016). Golgi staining was performed as described previously and above as in Aim 1 (Frankfurt et al., 2011; Kim et al., 2019; Taxier et al, submitted; Tuscher et al., 2016a) using the Rapid Golgi Stain Kit (FD Neuro Technologies).

As in aim 1, secondary basal dendrites and tertiary apical dendrites were counted from pyramidal neurons in the dorsal hippocampal CA1 and layer II/III of the 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.

Statistical analyses

All statistical analyses were conducted using GraphPad Prism 9 software (La Jolla, CA). In order 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 and 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 $p < 0.10$.

Results

Object Recognition

To determine whether *APOE* genotype influences the ability of E_2 to facilitate memory for a previously seen object or location, OVXed female E3FAD, E3/4FAD, and E4FAD mice (n = 11-21/group) were tested in the OR and OP tasks after receiving a post-training bilateral dorsal hippocampal infusion of vehicle or E_2. Our published data demonstrate that wild type C57BL/6 mice remember a previously seen object 24 h later (OR) and location 4 h later (OP) (Fortress et al., 2013; Taxier et al., 2019; Tuscher et al., 2018). In addition, we showed in the second chapter of this dissertation that intact untreated female E3FADs and E4FADs are memory-impaired at these delays (Taxier et al, submitted). In the present study, we chose delays of 24 h (OR) and 4 h (OP) test whether E_2 treatment can restore OR and OP memory to levels similar to wild type mice.

In the object recognition task, E_2-treated E3FADs (Fig. 12A; $t_{(15)} = 3.406$, $p = 0.004$) and E3/4FADs (Fig. 12A; $t_{(10)} = 2.745$, $p = 0.0201$) 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, vehicle-treated E3FADs, E3/4FADs, and E4FADs, as well as E_2-treated E4FADs, did not spend significantly 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.

12A; $F_{(1, 89)} = 9.614$, $p = 0.003$), 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 seemed to be driven by the beneficial effect of E_2 in E3FADs and E3/4FADs, given that vehicle-treated E4FADs and E_2-treated E4FADs spent similar amounts of time with the novel object during testing. Planned *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 between-group difference for vehicle-treated E3/4FADs versus E_2-treated E3/4FADs ($p < 0.031$), and a trend for a difference between vehicle-treated E3FADs and E_2-treated E3FADs ($p = 0.07$).

Interestingly, time to accumulate 30 seconds of object exploration during testing was significantly modulated by genotype (Fig 12C; $F_{(2, 86)} = 11.93$, $p < 0.0001$), such that E4FADs took significantly less time to reach 30 seconds 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 (Fig 12C; $p = 0.002$ vs E_2-treated E3FADs, and $p = 0.0004$ vs E_2-treated E3/4FADs).

Object Placement

The object placement task yielded similar results to the object recognition task, in that E_2-treated E3FADs (Fig. 12B; $t_{(12)} = 3.741$, $p = 0.003$) and E3/4FADs (Fig. 12B; $t_{(10)} = 5.644$, $p = 0.0002$) spent significantly more time than chance (15 s) with the moved object during testing, indicating intact memory for the identity of the training objects. Furthermore, vehicle-treated E3FAD, E3/4FAD, and E4FADs, as well as E_2-treated E4FADs, did not spend significantly more time than chance with the moved object during

testing, again suggesting that *APOE4* homozygotes are unresponsive to the mnemonic benefits of E_2. As with OR, a two-way ANOVA revealed a main effect of treatment (Fig. 12B; $F_{(1, 88)} = 8.183$, $p = 0.005$), as well as a significant genotype x treatment interaction (Fig. 12B; $F_{(2, 88)} = 3.591$, $p = 0.032$). However, unlike OR, there was also a significant main effect of genotype (Fig. 12B; $F_{(2, 88)} = 10.72$, $p < 0.0001$), such that E3FAD and E3/4FADs had superior memory for moved objects relative to E4FADs. The main effects of treatment and the significant genotype x treatment interaction seemed to be driven by the beneficial effect of E_2 in E3 and E3/4FADs, given that vehicle-treated E4FADs and E_2-treated E4FADs spent similar amounts of time with the novel object during testing. The main effect of genotype appeared to be driven by the overall poor performance of E4FADs, regardless of treatment. In support of these conclusions, planned *post hoc* comparisons indicated that E_2-treated E3FADs spent significantly more time with the novel object than E_2-treated E4FADs (Fig. 12B; $p = 0.002$). Similarly, E_2-treated E3/4FADs spent significantly more time with the novel object than E_2-treated E4FADs (Fig. 12B; $p = 0.0009$).

Similarly to OR, time to accumulate 30 seconds of object exploration during testing was significantly modulated by genotype (Fig 12D; $F_{(2, 89)} = 8.765$, $p = 0.0003$), such that E4FADs took significantly less time to reach 30 seconds of object exploration relative to other groups. Again, as with OR, the effect of genotype on time to complete the task was particularly driven by the rapid task completion of E_2-treated E4FADs (Fig 12D; $p = 0.007$ vs E_2-treated E3FADs).

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

E4FAD homozygosity may contribute to a hyperactivity phenotype in OVXed females, given that E4FADs completed the object tasks more quickly than EFADs of other genotypes. The observed increased activity in E4FADs from the present experiment is also consistent with the increase in speed seen in the OF task in the previous chapter.

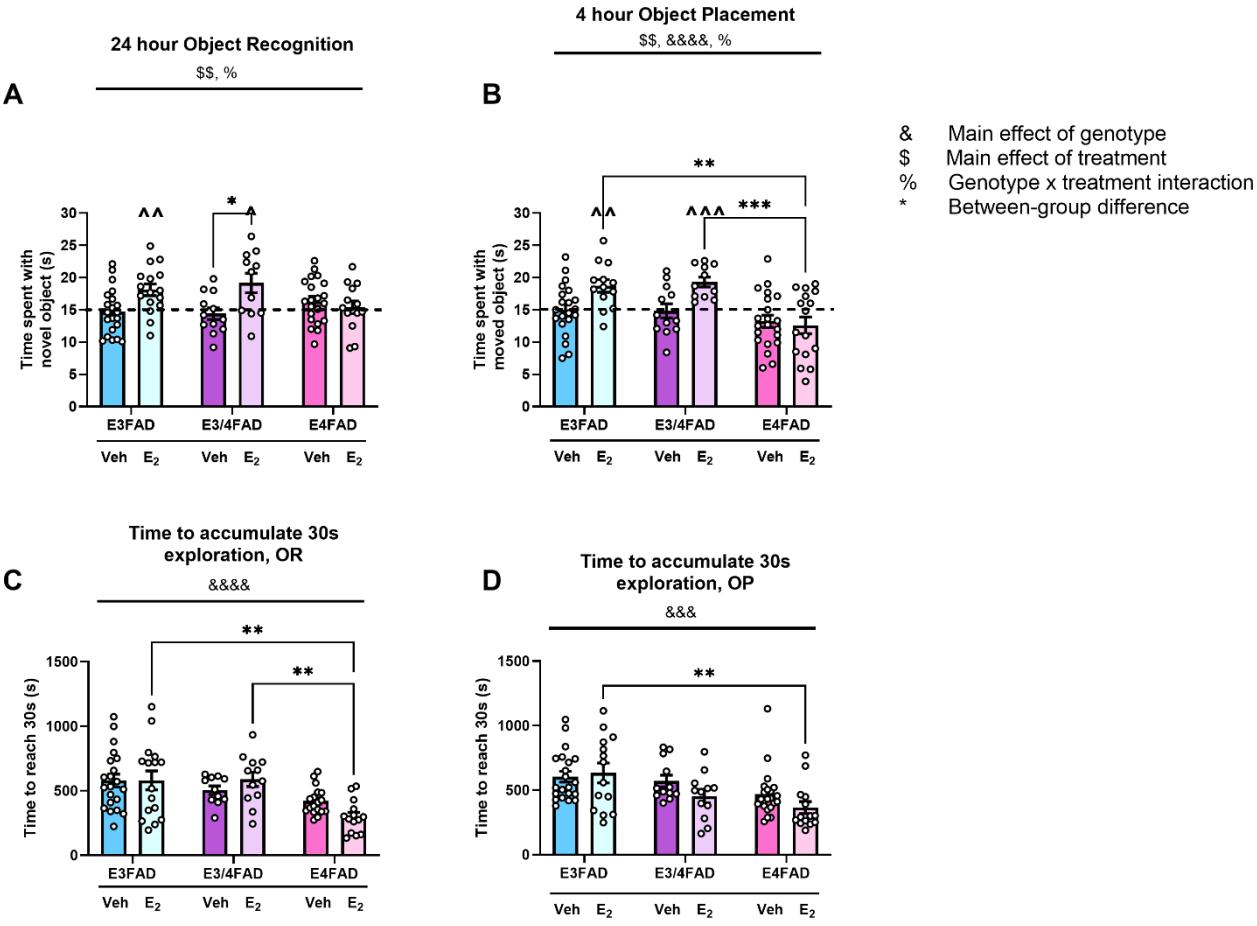


Fig.12 E_2 facilitates object recognition and spatial memory formation in OVXed E3FADs and E3/4FADs. E3FADs and E3/4FADs treated with E_2 spent significantly more time than chance (dashed line at 15 sec; ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$) with the novel (A) and moved (B) objects during testing. E_2-treated EFADs spent more time than veh-treated EFADs with the novel (A) or moved object (B) ($^{\$\$}p < 0.01$ = main effect of treatment; $^{\%}p < 0.05$ = genotype x treatment interaction). E_2-treated E3/4FADs spent more time with the novel object (A) than veh-treated E3/4FADs ($^{*}p < 0.05$). E3FADs and E3/4FADs spent more time with the moved object (B) than E4FADs ($^{\&\&\&\&}p < 0.0001$ = main effect of genotype). E_2-treated E3FADs spent more time with the moved object (B) than E_2-treated E4FADs ($^{**}p < 0.01$), as did E_2-treated E3/4FADs ($^{***}p < 0.001$). E4FADs took less time to complete either task (A, $^{\&\&\&\&}p < 0.0001$ = main effect of genotype; B, $^{\&\&\&}p < 0.001$ = main effect of genotype). E_2-treated E4FADs took less time than E_2-

treated E3/4FADs and E3FADs to accumulate 30s in OR (C; $^{**}p < 0.01$). E_2-treated E4FADs took less time to accumulate 30s in OP than E_2-treated E3FADs (D; $^{**}p < 0.01$) Bars represent mean ± standard error of the mean (SEM).

Western Blotting

Cell signaling and synaptic proteins

Because the memory-enhancing effects of E_2 are dependent upon the rapid activation of cell signaling cascades including ERK/MAPK and PI3K/Akt signaling within the DH, we examined expression of protein kinases and the synaptic protein PSD95, a downstream target of memory-related kinase activity (Yoshii & Constantine-Paton, 2014), in the DH of OVXed E3FAD, E3/4FAD, and E4FAD females 5 minutes after E_2 infusion. Significant results are reported below in the text, and nonsignificant data are reported in Table 4.

Two-way ANOVAs revealed a significant main effect of E_2 treatment, but no effect of genotype or treatment x genotype interaction on DH pPI3K expression (Fig. 13B; $F_{(1,50)} = 13.17$, $p = 0.0007$). Planned *post hoc* comparisons revealed that E_2-treated E3FADs had significantly less pPI3K than vehicle-treated E3FADs (Fig. 13B; $p = 0.018$). A significant main effect of E_2 treatment, while modest, was also present for pNR2B (Fig. 13C; $F_{(1,50)} = 4.216$, $p = 0.045$), in addition to a main effect of genotype (Fig. 13C; $F_{(2,50)} = 9.294$, $p = 0.0004$). Planned *post hoc* comparisons revealed that vehicle-treated E3FADs had significantly more pNR2B than vehicle-treated E4FADs (Fig. 13C; $p = 0.001$).

Table 4. Proteins in CA1 unaffected by E_2 treatment or APOE genotype

Protein	E3FAD		E3/4FAD		E4FAD	
	Veh	E_2	Veh	E_2	Veh	E_2
pERK42	100.0 ± 10.56	93.21 ± 10.05	109.39 ± 20.88	112.559 ± 16.44	109.447 ± 15.776	110.17 ± 14.46
pERK44	100.0 ± 9.32	119.5 ± 8.41	97.37 ± 12.2	124.85 ± 9.37	133.63 ± 18.73	111.44 ± 9.96
pJNK46	100.0 ± 2.05	89.52 ± 3.88	93.15 ± 6.28	94.97 ± 4.5	102.83 ± 5.25	94.02 ± 4.6
pJNK56	100.0 ± 5.86	89.7 ± 7.51	96.39 ± 8.67	90.6 ± 7.57	97.68 ± 7.94	87.35 ± 8.05
pβcatenin	100.0 ± 6.88	94.92 ± 6.73	109.42 ± 10.78	95.9 ± 8.73	94.29 ± 10.64	91.11 ± 5.52
pCofilin	100.0 ± 3.62	85.02 ± 8.66	78.35 ± 15.64	89.64 ± 16.62	96.08 ± 12.12	98.84 ± 13.47
pGSK3β	100.0 ± 7.91	100.09 ± 11.04	97.95 ± 4.56	98.2 ± 10.44	107.41 ± 9.13	93.69 ± 7.85
pPKC	100.0 ± 9.79	91.15 ± 9.06	97.99 ± 6.85	94.95 ± 4.43	101.8 ± 10.52	96.97 ± 5.44
Iba1	100.0 ± 11.01	134.75 ± 24.5	109.94± 9.58	112.39 ± 18.71	100.63 ± 9.61	117.52 ± 17.78
GPER	100 ± 4.43	92.62 ± 8.15	85.54 ± 9.42	85.47 ± 10.92	81.79 ± 7.11	88.86 ± 6.58
ERβ	100 ± 4.57	94.71 ± 8.32	109.64 ± 6.68	97.91 ± 4.36	98.69 ± 6.38	100.54 ± 5.89

* All proteins normalized to vehicle-treated E3FADs, whose values were set to 100.

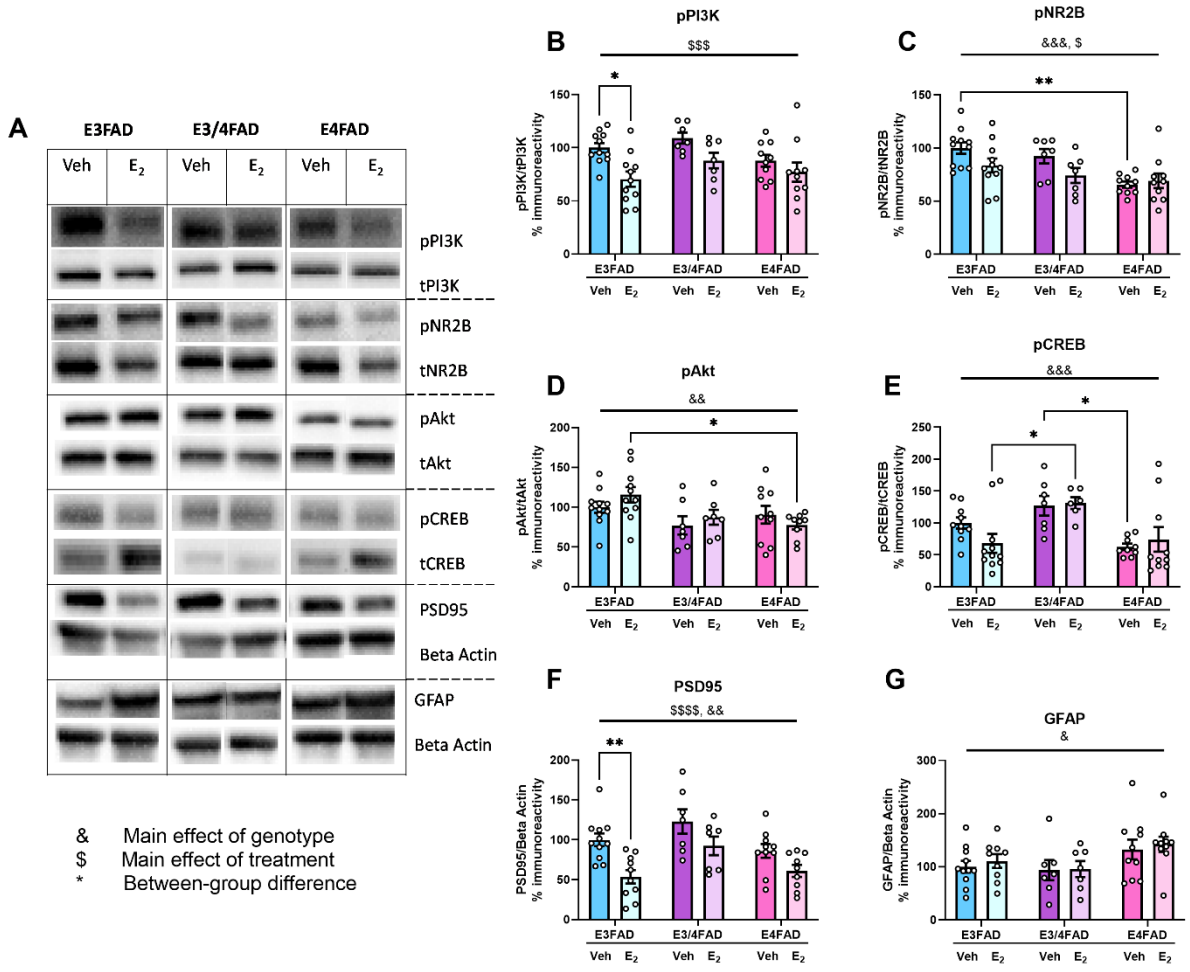
Values represent the mean % immunoreactivity (± SEM) for the target protein divided by β-actin (Iba1, GPER, ERβ) or unphosphorylated total protein (all phospho proteins).

There was main effect of genotype, but not treatment, nor an interaction, for pAkt (Fig. 13D; $F_{(2,50)} = 9.294$, $p = 0.0004$), which was in part driven by elevated pAkt in E_2-treated E3FADs relative to E_2-treated E4FADs (Fig. 13D; $p = 0.03$). Similarly, there was a main effect of genotype, but not treatment, nor an interaction, for pCREB (Fig. 13E; $F_{(2,47)} = 8.81$, $p = 0.0006$), which was oddly driven by elevated levels of pCREB in both vehicle-treated E3/4FADs (Fig. 13E; $p = 0.033$ vs vehicle-treated E4FADs) and E_2-treated E3/4FADs (Fig. 13E; $p = 0.041$ vs E_2-treated E3FADs).

Lastly, a two-way ANOVA revealed main effects of genotype (Fig. 13F; $F_{(2,48)} = 6.582$, $p = 0.003$) and treatment (Fig. 13F; $F_{(1,48)} = 18.03$, $p < 0.0001$), but no genotype x treatment interaction, on levels of DH PSD95. Planned *post hoc* comparisons revealed that vehicle-treated E3FADs had significantly more PSD95 than E_2-treated E3FADs (Fig. 13F; $p = 0.008$).

Glial proteins

Mounting evidence suggests that glial dysfunction is a prominent feature of AD (Heneka et al., 2015). Therefore, we looked at levels of glial proteins in the DH of EFAD mice following E_2 treatment. There was a significant main effect of genotype on DH levels of the astrocytic protein GFAP (Fig. 13G; $F_{(2,48)} = 4.232$, $p = 0.02$) such that E4FADs had higher levels of GFAP than mice of other genotypes, but no *post hoc* comparisons were significant. There were no significant main effects for the microglial protein Iba1 (Table 4).



<u>Fig. 13</u> E_2 treatment and *APOE* genotype modulate protein expression in the DH of OVXed EFAD mice. (A) Representative blots illustrate relative group differences in protein expression. Levels of pPI3K (A), pNR2B (C), and PSD95 (F) were decreased following E_2 treatment ($^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$, $^{\$\$\$\$}p < 0.0001$ = main effect of treatment). Levels of pNR2B (C), pAkt, and PSD95 (F) were decreased in E4FADs relative to other genotypes ($^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ = main effect of genotype). Levels of pCREB (E) were higher in E3/4FADs relative to other groups ($^{\&\&\&}p < 0.001$ = main effect of genotype). Levels of GFAP were higher in E4FADs relative to other groups ($^{\&}p < 0.05$ = main effect of genotype). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ = between-group differences.

Estrogen receptors

Because we previously saw that *APOE4* increased ERα expression in the DH of intact EFADs in the experiments described in the second chapter of this dissertation, we aimed to characterize expression of this protein, and other estrogen receptors, in OVXed EFADs treated with E_2. A two-way ANOVA revealed a significant main effect of genotype on levels of ERα (Fig. 14B; $F_{(2,48)} = 6.956$, $p = 0.002$), driven by elevated ERα in E4FADs relative to vehicle-treated E3FADs ($p = 0.052$ vehicle-treated E3FADs vs vehicle-treated E4FADs; $p = 0.019$ vehicle-treated E3FADs vs E_2-treated E4FADs). There were no significant differences in DH protein expression of either ERβ or GPER (Table 4).

Collectively, Western blot results suggest that both E_2 treatment and genotype differentially affect protein expression in the DH of EFAD mice, although they do not interact to modulate protein expression of any of the measured markers. Contrary to our hypothesis, E_2 treatment did not increase levels of phosphorylated kinases or synaptic proteins in the DH. In fact, main effects of E_2 treatment on levels of pAkt, pNR2B, and PSD95 were the opposite of what was expected, in that E_2 treatment decreased levels of these markers. For the most part, genotype effects were more consistent with expectations in that levels of pNR2B, pAkt, and PSD95 appeared to be diminished in E4FADs relative to other groups. Additionally, E4FADs exhibited elevated levels of ERα relative to other genotypes, and although not significant, heightened GFAP, which is

consistent with data from Chapter 2 of the dissertation. Most surprisingly, levels of pCREB were increased in E3/4FADs relative to other genotypes.

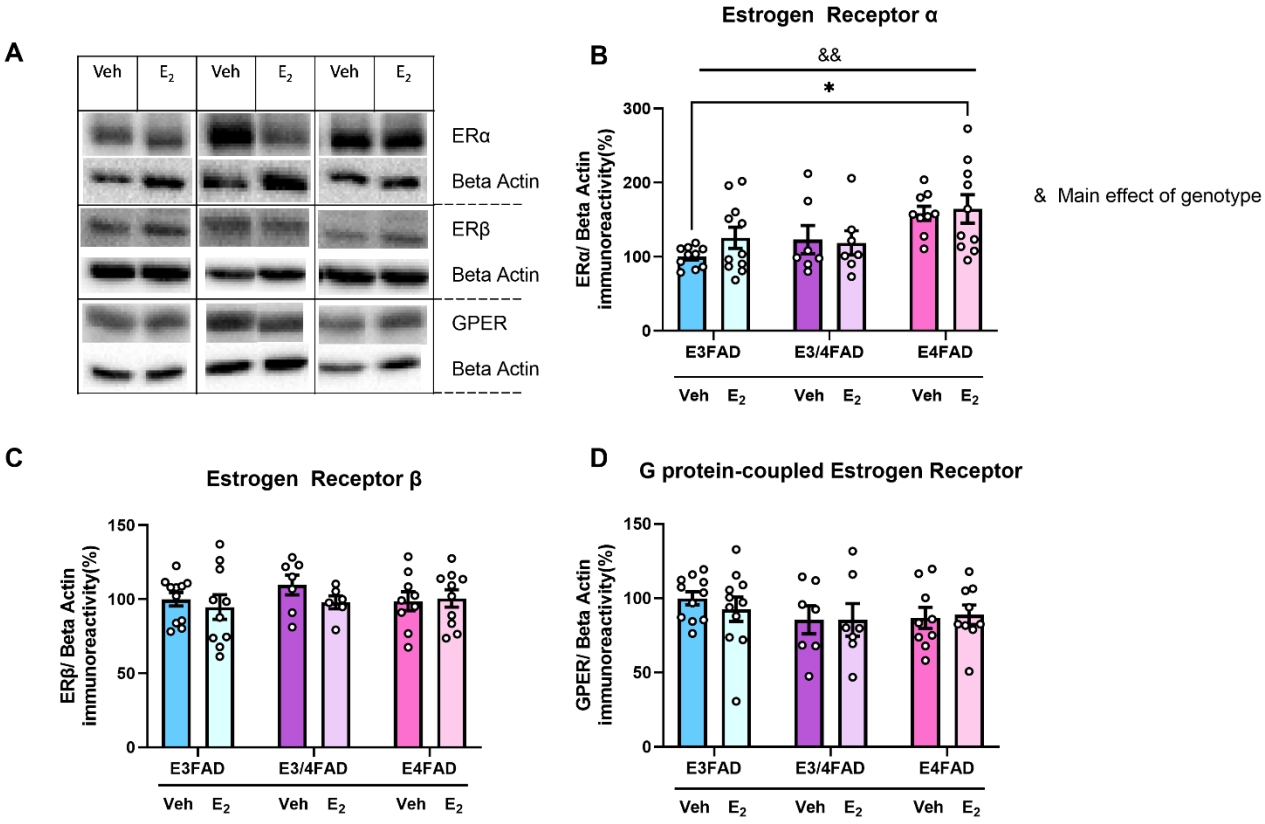


Fig 14. Expression of estrogen receptors in the DH of OVXed EFAD mice. Only ERα (B) was affected by *APOE* genotype ($^{\&\&}p < 0.01$ = main effect of genotype). Vehicle-treated E3FADs had significantly less ERα than E_2-treated E4FADs ($^{*}p < 0.05$). Levels of ERβ and GPER were unaffected by treatment or genotype.

Dendritic Analyses

We next examined apical and basal dendritic spine density in hippocampal area CA1 and in the prelimbic/infralimbic area of the mPFC to assess whether *APOE* genotype influences the ability of E_2 to increase dendritic spine density 2 h following DH infusion. There were no effects of genotype or E_2 treatment, nor an interaction, on mPFC apical

spines (Table 5). Treatment and genotype effects on CA1 apical, and CA1 and mPFC basal spines, are detailed below.

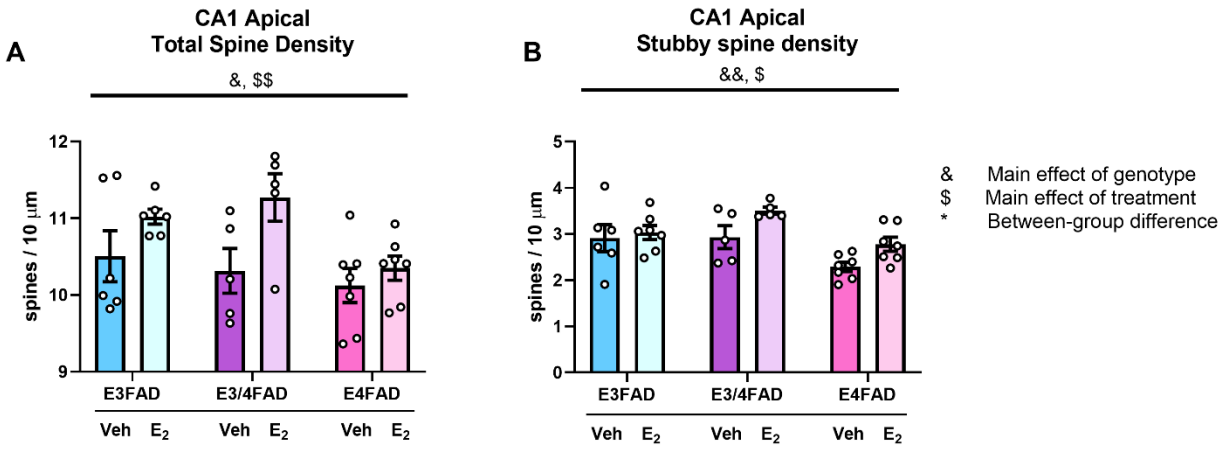


Fig 15. Intrahippocampal E_2 increases CA1 apical spine density in OVXed E3FAD and E3/4FAD mice. Total CA1 apical dendritic spine density (A) was increased 2 h following E_2 infusion into the DH ($^{\$\$}p < 0.01$ = main effect of treatment) and was diminished in E4FADs relative to other groups ($^{\&}p < 0.05$ = main effect of genotype). E_2 increased CA1 apical stubby spines (B), whereas E4FAD genotype diminished stubby spines ($^{\$}p < 0.05$ = main effect of treatment, $^{\&\&}p < 0.01$ = main effect of genotype)

CA1

A two-way ANOVA revealed significant main effects of genotype (Fig. 15A; $F_{(2,30)} = 3.635$, $p = 0.039$) and E_2 treatment on CA1 apical spine density (Fig. 15A; $F_{(1,30)} = 8.220$, $p = 0.008$), but no genotype x treatment interaction. Further analysis by spine subtype revealed no significant effects of genotype or treatment, nor interactions, on CA1 apical thin or mushroom spines (Table 5). However, there was a significant main effect of genotype (Fig. 15C; $F_{(2,31)} = 7.374$, $p = 0.002$), and of treatment (Fig. 15C; $F_{(1,31)} = 7.09$, $p = 0.012$) on CA1 apical stubby spines.

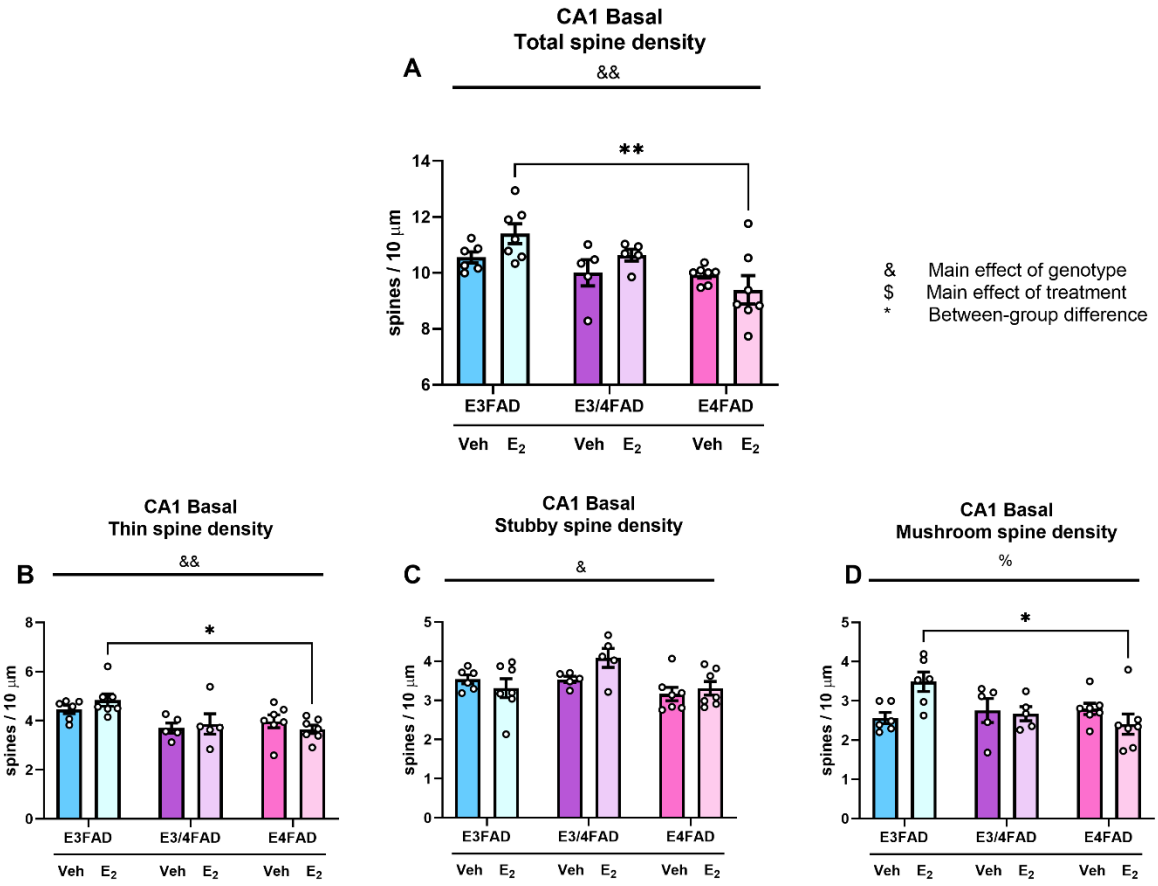


Fig 16. OVXed E4FADs have lower CA1 basal spine density than other EFADs. Total CA1 basal spine density (A), basilar thin spine density (B), and basilar stubby spine density (C) was decreased in E4FADs, regardless of treatment ($^{\&}p < 0.05$, $^{\&\&}p < 0.01$ = main effect of genotype). E_2-treated E3FADs had more mushroom spines than E_2-treated E4FADs ($^{\%}p < 0.05$ = significant genotype x treatment interaction). ($^{*}p < 0.05$, $^{**}p < 0.01$ = significant between-group differences).

A two-way ANOVA revealed a significant main effect of genotype (Fig. 16A; $F_{(2,31)} = 7.774$, $p = 0.002$), but no significant effect of treatment nor a genotype x treatment interaction on CA1 basal spine density. The main effect of genotype was primarily driven by elevated dendritic spine density in E_2-treated E3FADs, who had significantly higher basal spine density than E_2-treated E4FADs (Fig. 16A; $p = 0.002$). 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 thin spines (Fig. 16B; $F_{(2,31)}$ = 7.887, p = 0.002) and stubby spines (Fig. 16C; $F_{(2,31)}$ = 4.49, p = 0.019). The significant effect of genotype on CA1 thin basal spine density was driven by elevated thin basal spines in E_2-treated E3FADs relative to E_2-treated E4FADs (Fig. 16B; p = 0.013). There was a significant genotype x treatment interaction for levels of CA1 basal mushroom spines (Fig. 16D; $F_{(2, 30)}$ = 5.151, p = 0.012), which was driven by significantly more mushroom spines in E_2-treated E3FADs relative to E_2-treated E4FADs (Fig. 16D; p = 0.012).

Table 5. 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	4.10 ± 0.27	4.46 ± 0.33	4.06 ± .28	4.02 ± 0.11	4.26 ± 0.27	4.32 ± 0.21
	Apical mushroom	3.45 ± 0.34	3.23 ± 0.34	3.30 ± 0.23	15.6 ± 0.38	3.52 ± 0.78	3.20 ± 0.11
mPFC	Apical total	11.6 ± 0.88	10.35 ± 0.46	11.43 ± .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.

Combined, dendritic spine density analyses from CA1 of EFADs treated with E_2 suggest that E_2 may exert its memory-enhancing effects via increased CA1 apical spine density, particularly via its effects on apical stubby spines. The significant effect of genotype on CA1 basal spines mirrors the significant effect of genotype on CA1 basal spines seen in the experiments described in Chapter 2 of this dissertation, in that E4FADs exhibited markedly decreased basilar dendritic spine density relative to E3FADs. The genotype x treatment interaction effect on CA1 basal mushroom spines raises the important possibility that E_2 facilitates memory via increased CA1 basal mushroom spine density in E3FADs. Conversely, E4FAD homozygosity may promote an E_2-induced decrease in basilar mushroom spine density, and a corresponding decrease in cognitive capacity.

mPFC

A two-way ANOVA revealed a trend towards a significant main effect of genotype (Fig. 17A; $F_{(2, 30)} = 3.178$, $p = 0.055$), but not treatment nor a genotype x treatment interaction, for total mPFC basal spines. As determined by subsequent analyses on dendritic spine subtypes, this trend towards a main effect of genotype on total mPFC basal spines was driven by a significant main effect of genotype on basal mPFC thin spines (Fig. 17B; $F_{(2, 30)} = 5.212$, $p = 0.011$). 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 (Table 5) or mushroom spines (Table 5).

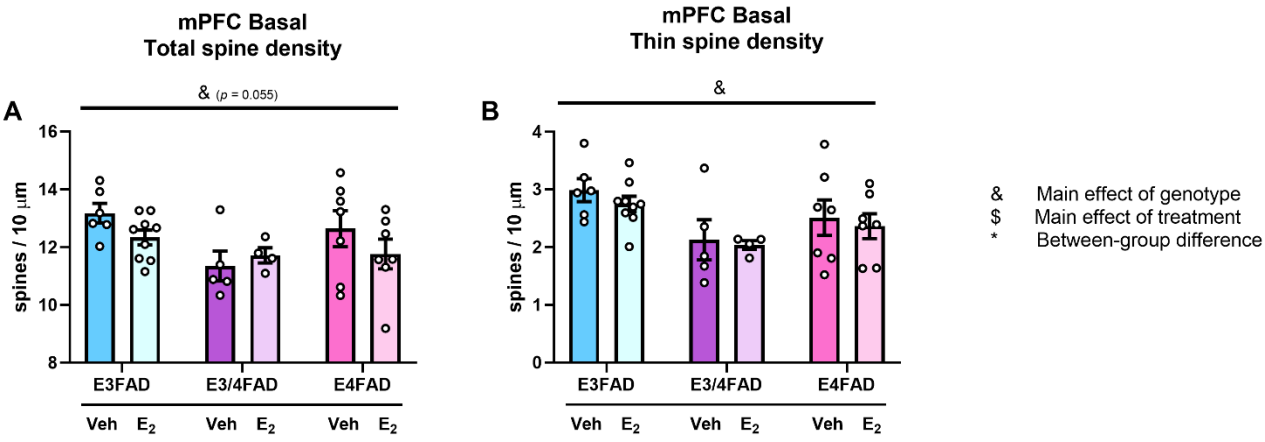


Fig. 17. E3FADs have more mPFC basal spines than E3/4FADs and E4FADs. mPFC basal spines (A; $^{\&}p = 0.055$) and thin spines (B) were modestly impacted by genotype (B; $^{\&}p < 0.05$).

The lack of genotype or treatment effects on mPFC apical spine density (Table 5), and modest genotype effects on mPFC basal spine density indicate that the memory-enhancing effects of E_2 on E3FADs and E3/4FADs are not mediated through changes in mPFC spine density. The modest effect of genotype on mPFC basal spines, both total and thin, suggests that E3FADs have heightened levels of basal spines relative to E4FADs, which is consistent with our findings in experiments described in Chapter 2 of this dissertation.

Discussion

Because *APOE4* status and female sex are unmodifiable risk factors that act synergistically to increase AD risk, 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 protein expression, and increase dendritic spine density in an EFAD mouse model of AD designed to model *APOE*-associated disease risk. We hypothesized that E_2 would have the most profound effects in OVXed E3FADs and E3/4FADs such that DH infusion of E_2 would promote memory consolidation, activate cell signaling cascades known to be involved in the memory-enhancing effects of E_2, and increase dendritic spine density in both the mPFC and DH. We also hypothesized that E_2 would either have reduced or no efficacy in OVXed E4FADs. Consistent with this hypothesis, we found that DH infusion of E_2 facilitated object memory consolidation in OVXed E3FADs and E3/4FADs, but not E4FADs, indicating that two copies of the *APOE4* allele results in lower susceptibility to the memory-enhancing effects of E_2. The memory-enhancing effects of E_2 were associated with an increase in total CA1 apical spine density. However, the memory-enhancing benefits of E_2 were not linked to an increase in cell-signaling kinase phosphorylation as predicted; indeed, in some instances E_2 decreased kinase phosphorylation or had no effect, regardless of *APOE* genotype. *APOE4* genotype alone, in the absence of an E_2 effect, modulated several spine parameters and levels of proteins in the DH. We observed only a single genotype x treatment interaction, suggesting that E_2 increases basilar mushroom spines in CA1 in E3FADs, but decreases this subtype in E4FADs.

Although others have shown a potential benefit for chronic E_2 treatment for decreasing amyloid accumulation in the EFAD model (Kunzler et al., 2014) and in the 3xTg model of AD (Carroll et al., 2007), our data are the first to demonstrate that a single acute dose of E_2 can facilitate memory consolidation in OVXed E3FAD and E3/4FAD, but not E4FAD mice. Previous work demonstrated that chronic E_2 treatment initiated in 3-

month-old OVXed EFADs can significantly reduce amyloid burden (Kunzler et al., 2014). Our finding that E3FAD and E3/4FAD mice remain susceptible to the beneficial effects of a single acute dose of E_2 until at least 6 months of age, when there is already substantial development of AD-like pathology (Tai et al., 2017), is particularly striking. Whether *APOE4*-negative patients with AD can benefit from E_2 treatment after substantial disease progression remains an important question.

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 & 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). More work remains to determine what factors mediate the neuroprotective properties of E_2 in *APOE4* carriers.

Protein expression data from the present study did not mirror the beneficial effects of E_2 on memory consolidation. For instance, levels of protein kinase phosphorylation were either opposite of what was hypothesized or unchanged in response to treatment with E_2. For example, levels of pPI3K, pNR2B, and PSD95, all of which are activated by E_2 in young wild type mice (Akama & McEwen, 2003; Fan et al., 2010; Jie et al., 2018), were reduced by E_2 in the present experiment, particularly in E3FAD and E3/4FAD mice. One possible explanation for these findings is that E_2 is functioning to depress

hyperexcitability or abnormally high kinase activity in E3FAD and E3/4FAD mice. However, given that E_2 treatment reduced levels of these proteins to amounts akin to those present in E4FADs, regardless of treatment status, this possibility seems unlikely. Another potential explanation for an E_2-induced depression of phosphorylated kinase activity is that levels of total protein kinases are different across genotypes, which we did not capture because we looked at the ratio of phosphorylated protein relative to the total protein. Additional analyses directly comparing expression levels of the total form of each protein kinase normalized to Beta Actin or another housekeeping protein could answer whether levels of total protein kinase are different across genotypes. This type of analysis could also aid in interpreting the effects of *APOE* genotype and E_2 treatment on levels of pCREB, which were confusingly high in E3/4FADs, regardless of treatment status, relative to other groups. Another possibility explaining the lack of an E_2 effect on several kinases is that the dynamics of protein kinase phosphorylation are distinct in EFAD mice from what we typically see in healthy young wild-type mice. That is, perhaps the initiation of protein kinase phosphorylation is slower, or more transient, in the EFAD brain. Taking tissue from these mice at multiple time points post E_2-infusion, rather than the single time point taken here, would help elucidate the time course of kinase phosphorylation events in the EFAD model.

Genotype effects on levels of hippocampal proteins were more consistent with expectations, in that E4FADs had elevated GFAP and ERα expression relative to other genotypes, as in experiments from Chapter 2 of the dissertation. These data suggest that E4FADs may be rendered less susceptible to the beneficial effects of E_2 because of elevated astrogliosis. Interestingly, although E_2 interacts with ERα to facilitate memory

consolidation in healthy young wild-type mice (Boulware et al., 2013), here it seems that elevated ERα may have a negative effect on memory consolidation. Consistent with our own finding, others have shown that ERα is elevated in the hippocampus of AD patients, particularly *APOE4* carriers (Ishunina & Swaab, 2003).

Although E_2 treatment effects on hippocampal protein levels were unclear and contradictory to our hypotheses, dendritic spine data were more consistent with the behavioral effects in the present study. In particular, E_2 increased CA1 apical spine density, an effect that, while not significant by *post hoc* comparisons, was most pronounced in E3FAD and E3/4FADs. The E_2-facilitated increase in CA1 apical dendritic spine density in the present study is consistent with previous research from our lab showing that CA1 apical spine density is increased 2 hours following E_2 infusion in young wild-type OVXed female mice (Tuscher et al., 2016). We also showed that E_2 increased CA1 apical stubby spine density in EFADs. Although thin spines are typically considered to be more dynamic, previous work in another AD model suggests that stubby spines take on a more dynamic role in pathological conditions (Spires-Jones et al., 2007). Therefore, the 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 2 hours post intrahippocampal E_2-infusion in young wild-type mice (Tuscher et al., 2016). However, we did not see a corresponding increase in CA1 and mPFC basal spines following E_2 infusions in E3FADs or E3/4FADs as in wild-type mice, which may be reflective of reduced interactions or connectivity between mPFC and CA1 in E_2-treated EFADs.

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 relative to other groups, 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 or "memory" spines (Bourne & Harris, 2007).

The most striking of our observed effects on dendritic spine density was our finding of a sex x treatment interaction on CA1 basal mushroom spines. This finding, in which E_2-treated 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 & Harris, 2007; Hayashi & Majewska, 2005), we posit that the beneficial effects of E_2 on memory in E3FADs are linked to this increase in CA1 mushroom spines. Although the same effect of E_2 on CA1 basal mushroom spines was not present in E3/4FADs at the single time point we examined, it may be the case that the single copy of *APOE4* in E3/4FADs slowed the spine remodeling process, which is highly dynamic, and an E_2-facilitated increase in mushroom spines would have occurred

on a slower time scale in these mice. In addition, low sample size and high within-group variability in the E3/4FAD groups could also have occluded the potential benefits of E_2 on basilar CA1 mushroom spines in E3/4FADs.

In sum, results from the current study suggest that intrahippocampal E_2 infusion can facilitate memory consolidation and correspondingly increase CA1 apical spine density in E3FADs and E3/4FADs. Notably, *APOE4* genotype had a deleterious effect on the ability of E_2 to enhance memory, and also increased levels of GFAP and ERα in a manner presumably linked to impaired memory consolidation. In addition, a beneficial effect of E_2 for E3FADs, and a detrimental effect of E_2 for E4FADs, on CA1 basal mushroom spines may account for the respective ability and inability of E_2 to enhance memory in E3FADs versus E4FADs. As with the experiments described in the second chapter in this dissertation, this study is limited by the examination of protein and spine density correlates of the mnemonic benefits of E_2 at a single time point in EFAD mice. Therefore, future experiments should examine the kinetics of both protein expression and dendritic spine density following E_2 treatment at multiple time points. Nevertheless, these data support the notion that E_2 treatment can potentially benefit individuals who carry one copy of the *APOE4* allele. More work remains to fully characterize the interactive effects of E_2 and *APOE4* genotype in service of developing targeted treatment strategies for AD patients.

CHAPTER FIVE: Summary, significance, and future directions

In summary, the findings of this dissertation have added to a growing body of literature detailing the detrimental effects of *APOE4* genotype on memory consolidation, an effect that is exacerbated in females. Observed *APOE4*-associated deficits in object memory consolidation were linked to a detrimental impact of *APOE4* on dendritic spine density in CA1 and in mPFC, and protein alterations in the dorsal hippocampus. We also showed a detrimental impact of *APOE4* and of ovariectomy on anxiety-like behavior in the open field. Finally, we showed that E_2 treatment facilitated memory in ovariectomized *APOE3* carriers and carriers of a single copy each of *APOE3* and *APOE4*, but not in *APOE4* homozygotes. The beneficial effects of E_2 were linked to a corresponding increase in CA1 apical dendritic spine density. By leveraging EFAD mice to model *APOE*-associated disease risk, we have provided valuable preclinical evidence that sex, *APOE* genotype, and estrogens are crucial interacting factors contributing to AD-related risk and outcomes.

Clinical trial failure rates for AD therapies are astoundingly high, illustrating the indisputable fact that we urgently need better treatments for AD. Furthermore, targeted therapies that account for unmodifiable risk factors like *APOE* status, age, and sex, are sorely lacking. Thus, much more preclinical work needs to be done to elucidate relationships among these risk factors and develop interventional approaches to slow and treat disease progression.

Work from this dissertation illustrates the complex nature of interactions among variables like *APOE* genotype and sex, as described in Chapter 2, or *APOE* status and estrogens, as described in Chapter 4. Although our primary aim in this dissertation was

to characterize interactions between *APOE* genotype and sex, or *APOE* genotype and estrogens, we found surprisingly few interactions between these variables. Because we primarily found effects of *APOE* genotype, and not interactions between genotype and sex or genotype and estrogens, several questions remain about how *APOE* genotype interacts with these variables in the EFAD model to contribute to disease progression.

One potential future direction that may capture the interactive relationship between *APOE* and sex would be to evaluate memory consolidation, dendritic spine density, and protein alterations at an earlier age in EFADs. Because EFADs develop substantial pathology by 6 months of age, it may have been the case that potential interactions were occluded by severe impairment in EFADs of all groups apart from male E3FADs. Additionally, object tasks may not be the most suitable for detecting *APOE* x genotype interactions, because they rely on a single measurement of memory consolidation (time spent with either the moved or novel object during testing). Although we did not see differences in Morris water maze performance, we suspect that this is due to the level of task difficulty. Because EFADs, regardless of sex or genotype, could rapidly learn the location of the hidden platform in the water maze, a more challenging water maze, with fewer or less obvious spatial cues, may be better suited for identifying sex x genotype interactions. Although we hypothesized that *APOE* genotype and sex might interact to affect levels of hippocampal proteins we know to be important for memory processes, we found no interactive effects of *APOE* genotype and sex on hippocampal protein levels in the proteins assayed. A quantitative mass-spectrometry approach could be a useful screening tool to characterize the hippocampal proteome in EFADs and to examine how it may be affected by genes and sex.

Our approach towards capturing the interactive effects of *APOE* status and estrogens was to examine factors that we know are critical for E_2-induced memory enhancement in young, wild-type mice. However, we were surprised to discover that the dorsal hippocampal protein expression profile of E_2-treated E3FADs and E3/4FAD is starkly different than that of wild-type mice 5 min following E_2 infusion. Therefore, one potential future direction would be to more fully characterize the time course of E_2 activation in EFADs by examining protein expression at multiple time points post-infusion. Although we assumed E_2 would exert its effects in EFADs via the same pathways it activates in wild type mice, we did not find this to be the case. Therefore, an extremely useful step would be to infuse E_2 into EFAD mice followed by a sequencing approach such as RNA-seq to identify potential targets of E_2 action in EFADs.

Finally, because Golgi staining only captures dendritic spine density at a single time point, employing chronic *in vivo* imaging approaches to track changes in dendritic spine density over time would provide extremely useful information about spine dynamics in the EFAD model, and could elucidate whether there are differences in spine dynamics that are impacted by sex, *APOE* genotype, or estrogens. Although chronic *in vivo* imaging of hippocampal spines is made difficult by the depth of the structure, chronic imaging of cortical spines would still yield novel data about spine dynamics in EFAD mice.

Although rodent models of AD have existed for many years, basic research has yet to identify what causes AD, and how to prevent it. Because there is no cure, AD places an enormous burden on healthcare providers, caregivers, and most importantly, patients. Deaths from AD have increased 145% between the years 2000 and 2019, and during the COVID-19 pandemic, deaths from AD have increased 16% (Alzheimer's Association,

2021). Because *APOE4* status and female sex converge to increase AD risk, examining these factors in concert is key for developing precision medicine approaches to treating AD. Leveraging the EFAD model to identify crucial interactions among the trifecta of unmodifiable risk factors for AD: age, *APOE4* status, and sex, could ultimately lead to the identification of targetable biomarkers for precision AD therapeutics.

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