EFAD transgenic mice as a human *APOE* relevant preclinical model of Alzheimer's disease

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Abbreviations: AA: arachidonic acid; Aβ: amyloid-β; AD: Alzheimer's disease; ABCA1/ABCG1: ATP-binding cassette transporter subfamilies A and G; ADAPT: Anti-inflammatory Prevention Trial; Alt-ET: estrogen therapy alternatives; APP: amyloid precursor protein; BBB: blood-brain barrier; BDNF: brain-derived neurotrophic factor; BACE1: β-secretase 1; CAA: cerebral amyloid angiopathy; cGMP: cyclic guanosine monophosphate; CM: cross-maze; CREB: cAMP-response element binding protein; CSF: cerebrospinal fluid; CTA: conditioned taste aversion; CVD: cerebrovascular dysfunction; CX: cortex; DHA: docosahexaenoic acid; E_2: 17-β estradiol; EGF: epidermal growth factor; ER: estrogen receptor; ET: estrogen therapies; FA: formic acid; FAD: familial AD; FC: fear conditioning; GFAP: glial fibrillary acidic protein; GWAS: genome-wide association study; h-*APOE*: human *APOE*; HP: hippocampus; Iba1: ionized calcium binding adaptor molecule 1; IHC: immunohistochemistry; IL-1β: interleukin 1 beta; IL-4R: interleukin-4 receptor; LPC: lysophosphatidylcholine; m-*APOE*: mouse *APOE;* MCI: mild cognitive impairment; mfsd2a: major facilitator superfamily domain-containing protein 2; MOAB-2: monoclonal antibody specific to amyloid-beta; mAb: monoclonal antibody; M: months; MWM: Morris water maze; NeuN: hexaribonucleotide binding protein-3; NFT: neurofibrillary tangles; NOR: novel object

recognition; NSAID: nonsteroidal anti-inflammatory drugs; NSE: neuron-specific enolase; oAβ: oligomeric conformation of amyloid-β; OVX: ovariectomized; PSD95: postsynaptic density protein 95; RXR: retinoid X receptor; SB: subiculum; pCREB: phosphorylated CREB; pTau: phosphorylated tau; PL: phospholipids; SEM: selective estrogen mimic; SERM: selective estrogen receptor modulator; TBSX: TBS + Triton X-100; Thio-S: thioflavine-S; Thy-1: thymocyte differentiation antigen 1 theta; Tg: transgenic; TLR4: toll-like receptor 4; TNFα: tumor necrosis factor alpha; TR: targeted-replacement; TREM2: triggering receptor expressed on myeloid cells 2; YM: Y-maze.

Abstract

Identified in 1993, *APOE4* is the greatest genetic risk factor for sporadic Alzheimer's disease (AD), increasing risk up to 12-fold compared to *APOE3,* with *APOE2* decreasing AD risk. However, the functional effects of *APOE4* on AD pathology remain unclear and, in some cases, controversial. *In vivo* progress to understand how the human (h)-*APOE* genotypes effect AD pathology has been limited by the lack of a tractable familial AD-transgenic (FAD-Tg) mouse model expressing h- rather than mouse (m)-*APOE*. The disparity between m- and h-apoE is relevant for virtually every AD-relevant pathway, including amyloid-β (Aβ) deposition and clearance, neuroinflammation, tau pathology, neural plasticity and cerebrovascular deficits. EFAD mice were designed as a temporally useful preclinical FAD-Tg-mouse model expressing the h-*APOE* genotypes for identifying mechanisms underlying *APOE*-modulated symptoms of AD pathology. From their first description in 2012, EFAD mice have enabled critical basic and therapeutic research. Here we review insights gleaned from the EFAD mice and summarize future directions.

Keywords: Alzheimer's disease, Animal Models, Lipoproteins, apolipoproteins, Brain, behavior, histopathology, neuronal viability, apoE lipidation, cerebrovascular dysfunction

Introduction

AD: Humans to Transgenic mice

In humans, Alzheimer's disease (AD) progresses over decades, resulting in synaptic dysfunction eventually leading to neuronal loss. Despite the large number of longitudinal aging studies in humans, the lack of cognitive measures coordinated with symptoms of AD pathology results in a poorly understood disease trajectory (1). Transgenic (Tg)-mice are a powerful tool to track AD pathology, address mechanistic hypothesis and assess the activity of potential therapeutics. An overall limitation of all Tg-mouse models is that none reproduce the full spectrum of human AD symptoms and pathology. However, a number of reviews provide specific guidelines for preclinical AD studies with a consistent theme that a useful Tg-mouse model will incorporate major human AD risk factors and demonstrate dysfunction in the AD-relevant symptom of interest (2). Thus, critical to this modeling is the inclusion of the major human AD risk factors. The *APOE4* genotype is the greatest genetic risk factor increasing AD risk up to 15-fold compared to the common *APOE3* genotype, while *APOE2* is protective (3, 4) but less frequent (estimated: ε2/2 at 0.4%, ε2/3 at 8.8% and ε2/4 at 1.5%; (5, 6)). Although this risk was identified in 1993 and a number of hypotheses propose *APOE* genotype-specific functions, the mechanistic pathways that cause *APOE4*-induced AD risk remain unclear. Even less understood is the critical link between female sex and the *APOE4*-induced AD risk (6-13). As described, the sequence of pathology in human AD patients is unclear, however there are key AD-relevant outcomes that are modulated by *APOE* and sex in humans. AD is diagnosed by extracellular amyloid plaques and neurofibrillary tangles (NFT) of tau. In addition, soluble oligomeric conformations of amyloid-β (oAβ) correlates with cognitive decline and disease severity (14-19). Importantly, oAβ levels in cerebrospinal fluid (CSF) are increased in AD patients vs. controls; and are greater with *APOE4/4* vs. *APOE3/3* in AD patients (20). Further pathology includes neuronal dysfunction, neuroinflammation, and cerebrovascular dysfunction (CVD). Hence, it is essential to study the interactive role of AD risk factors: age, sex and *APOE* genotype in the development of human AD pathology using mouse models.

Mouse APOE vs. human APOE in AD-Tg mouse models

In vivo progress to determine the effects of the human (h)-*APOE* genotypes on AD pathology has been limited by the lack of a tractable familial AD-Tg (FAD-Tg) mouse model expressing h- rather than mouse (m)-*APOE* (for complete review on the introduction of h-*APOE* into FAD-Tg mouse models see (21)). Why is this a critical issue? m-apoE is structurally and functionally distinct from h-apoE. While the three human isoforms of apoE differ by a single amino acid change at residues 112 and 158 (apoE2 ^{Cys,Cys}, apoE3^{Cys,Arg}, apoE4^{Arg,Arg}), m-apoE is expressed as a single isoform and differs from h-apoE by ~100/300 amino acids. The disparity between m- and h-apoE is relevant for virtually all AD-relevant pathology, including Aβ deposition and clearance, neuroinflammation, tau pathology, neural plasticity and CVD (22-29). Further, many *APOE*/FAD-Tg models express h-apoE under heterologous promoters that may be activated by stressors (GFAP: glial fibrillary acidic protein (27, 30)), or by cell types that are not the primary producers of apoE (NSE: neuron-specific enolase (31)), rather than the endogenous m-apoE promoter. Currently, the best option is the *APOE*-knock-in or targeted-replacement (TR) mice, developed to replace the coding domain for m-*APOE* with the coding domain of each of the h-*APOE* genotypes (32). In *APOE*-TR mice, glial cells express h-*APOE* in a native conformation at physiologically regulated levels, and in the same temporal and spatial pattern as endogenous m-*APOE*. Thus, it is critical to conduct mechanistic and preclinical therapeutic studies in mice that incorporate h-*APOE* in a relevant context.

Building an AD-Tg mouse model with human APOE

Operationally, relative to m-*APOE*, the h-*APOE* genotypes delay AD pathology in FAD-Tg mouse models, as illustrated in Table 1. This table is designed to be a representation, using a composite of several FAD-Tg mouse models (27, 33-46) and Aβ pathology as an example of AD phenotype. First, **Aβ pathology is significantly delayed** when FAD-Tg mice are crossed with *APOE*-knock-out (KO) mice, eliminating m-*APOE* (Table 1: compare A to B) (27, 30, 39, 40, 46). Second, **Aβ pathology is further delayed** when h-*APOE*-Tg mice are crossed with FAD-Tg/*APOE*-KO mouse (Table 1: compare B to C)

(28, 30, 47, 48). Thus, incorporation of h-*APOE* into FAD-Tg mice that normally begin to develop pathology at 8-10 months (M) delays the development of pathology to 12-16M (27, 28, 30, 47, 48), extending progression of the full array of AD-related symptoms beyond the lifespan of the mouse. One approach to address this h-*APOE*-induced temporal delay in AD pathology is using an FAD-Tg mouse that has rapid-onset AD pathology. 5xFAD mice exhibit accelerated development of pathology, including amyloid deposition by 2M (Table 1: compare A to D) (49-69). The 5xFAD mice were crossed with *APOE*-TR mice to produce the EFAD mice[f], which exhibit the anticipated delay in AD pathology, particularly with plaque development delayed to ~6M (Table 1: compare D to E) (20, 66, 70-75) but still >6M before the average FAD-Tg/*APOE*-Tg mice (compare Table 1: C and E).

Development of the EFAD mouse model

5xFAD mice co-express 5 FAD mutations (APP K670N/M671L + I716V + V717I and PS1 M146L + L286V) under the control of the neuron-specific mouse thymocyte differentiation antigen 1 theta (Thy-1) promotor (50). The specificity of this Thy-1 promoter for neuronal expression in the brain has been established (76, 77). The 5xFAD line Tg6799 that produce the highest amount of Aβ and are heterozygous for the 5xFAD genes was provided by Dr. R. Vassar (Northwestern University). Homozygous *APOE*-TR mice were purchased from Taconic in collaboration with Dr. P. Sullivan (Duke University) (32, 78). Details on the production, genotyping, and genetic background of these two mouse strains are described in the above papers. To establish colonies of EFAD mouse lines (E2FAD, E3FAD and E4FAD), 5xFAD mice were bred to homozygous *APOE*2-, *APOE*3- and *APOE*4-TR mice by Taconic labs[a]. Briefly, male *APOE*-TR$^{+/+}$ mice on a C57/B6 background were bred with female 5xFAD$^{+/-}$ mice on a C57B//B6xSJL background. The resulting female mouse- *APOE*/*APOE*-TR/5xFAD$^{+/-}$ mice were backcrossed with male *APOE*-TR mice to generate *APOE*-TR$^{+/+}$/5xFAD$^{+/-}$ (EFAD) mice. The **EFAD mice** allow for the monitoring of multiple AD-related symptoms through the mouse lifespan (Tables 2 and Table 3). Importantly, based on the design of the breeding strategy, the EFAD mice littermates (50%) are non-carriers (EFAD-NC) for the 5xFAD transgenes and homozygous for *APOE* (5xFAD$^{-/-}$/*APOE*$^{+/+}$).

Thus, EFAD-NC provide both a comparison to the EFAD mice and a complementary approach to the address functional questions about *APOE* in the absence of FAD-induced pathology.

Amyloid plaque deposition ≠ soluble Aβ peptide

Amyloid plaques (together with NFT) are a major pathological hallmark of AD, however there is strong debate on their significance. Understanding this issue is relevant for interpreting data obtained in EFAD mice. Recently, patience has grown thin in both the public and academic communities for the continued failure of both Aβ vaccine trials[b] and amyloid imaging studies (79-81). This has led to an apparent bias against amyloid plaques as a therapeutic target and even Aβ as a measure of AD pathology, evident from publications (82-85), press releases[c,d], documentaries[e] and TEDx talks[f]. One aspect of this debate is the nature of the aggregation pathways for Aβ, particularly Aβ42. As illustrated in Figure 1, one paradigm for considering this assembly process whether soluble oAβ is either "on" or "off" pathway to the assembly of amyloid plaques (86). Via the "on" pathway, oAβ can be pathogenic, but are structural precursors for the formation of the traditional insoluble fibrils that continue to assemble into the parallel β-sheet structure of amyloid plaques, also considered to be pathogenic (Figure 1A). Alternatively, in the "off" pathway assembly process, Aβ can proceed via two separate processes, the pathogenic formation of oAβ or formation of the traditional insoluble fibrils that continue to assemble into amyloid plaques, considered to be benign (Figure 1B). Whether the "on" or "off" pathway is correct has not yet been determined. However, both the vaccine trials and the imaging studies focus on amyloid plaques, not oAβ, as the pathogenic target. This distinction is critical as FAD mutations indicate only that increases in Aβ42 or the increase in the Aβ42/Aβ40 ratio cause FAD, not that the toxic assembly form of the peptide is amyloid plaques. Indeed, a novel FAD-Tg mouse model that promotes oAβ formation, rather than fibrils and amyloid plaques, develops features of AD pathology including tau hyperphosphorilation, neuroinflammation, synaptic alteration, cognitive deficits and neuronal loss (87). For the purposes of this

review, we consider that oAβ levels and induced signaling cascades are pathogenic, and amyloid plaques are likely, at best, less neurotoxic and more likely to be benign.

Phenotypic Characterization of EFAD Mice

5xFAD vs EFAD mice (Tables 2 and 3)

The EFAD mice are tractable mouse model to study the development of a number of components of AD pathology. Although 5xFAD express m-apoE, which we argue is distinct from human apoE in the proximal mechanistic pathways that lead to AD-pathology, we have included a comparison of AD pathology in 5xFAD vs. EFAD mice to demonstrate, in part, the rapid onset and accelerated development of AD symptoms in the 5xFAD (Table 2, Table 3). In addition, pathology in 5xFAD mice can be used to prediction pathology in the EFAD mice. A particularly useful example is neuronal loss, which is significant by 12M in the 5xFAD mice, predicting that in the EFAD mice neuronal loss will likely occur >16M. However, *APOE* genotype will significantly influence most components of AD pathology and analysis of these differences will illuminate *APOE*-modulation of AD pathology. In general, there is an *APOE* genotype (E4FAD > E3FAD ≥ E2FAD), as well as an emerging sex effect (female (♀) > male (♂)) on behavioral deficits, histopathology, and neuronal viability (Table 2), as well as Aβ42 and apoE solubility, neurotrophic factors and neuroinflammatory cytokines but not with amyloid precursor protein (APP) processing (Table 3). Thus, the **EFAD mice** allow for the monitoring of multiple AD-related symptoms through the mouse lifespan (Tables 2 and 3). An important note is that the sex and *APOE4* genotype interactions are recent findings, and ♂EFAD mice are more extensively characterized than ♀ mice. Here our goal is to detail *APOE* modulated AD pathology in EFAD mice, and, where applicable, ♀ vs. ♂ comparisons.

APOE modulated AD symptoms exhibited in EFAD mice (Figures 2-5)

Behavioral deficits in ♂ and ♀ EFAD mice

In humans, AD is characterized by progressive behavioral changes, including memory loss, a decrease in executive function, and impairments in social interaction (88-98). Cognitive decline in human AD patients (99) and normal population follows ε4>ε3>ε2 (100), greatest in ♀ε4 carriers (5, 101, 102). Similarly, in ♀EFAD mice, cognitive impairment is E4FAD > E3FAD ≥ E2FAD as measured by Morris water maze (MWM), with the deficits increasing from 2-6M (Figure 2A) (70). In 8M E3FAD and E4FAD mice, cognitive impairment is E4FAD > E3FAD and ♀>♂ as measured by novel object recognition (NOR) and Y-Maze (YM) (Figure 2B) (71). Although cognitive testing mice is not without drawbacks, it is striking that EFAD mice demonstrate *APOE* genotype- and sex-induced loss of cognitive function similar to humans.

Extracellular amyloid and Aβ in ♂EFAD mice

Human data demonstrate higher levels of extracellular amyloid/Aβ with *APOE4* compared to *APOE3* (20, 103, 104). For our initial characterization of the pathology in the EFAD mice, we used ♂ mice aged 2-6M (Figure 3) (66). Both Aβ accumulation and Thio-S-positive plaque deposition begins in the subiculum (SB), followed by the deep layers of the frontal cortex (CX) (Figure 3 A,B) then spreading to the outer layers of the CX and the thalamus (50, 66). As described above, introduction of h-*APOE* delayed extracellular Aβ accumulation from ~2-6M compared to the 5xFAD mice expressing m-*APOE*: 5xFAD > E4FAD > E3FAD ≥ E2FAD (Figure 3A). Plaque morphology is also affected by *APOE* genotype, with diffuse plaques: E2FAD = E3FAD > E4FAD and compact plaques: E2FAD = E3FAD < E4FAD (66).

Neuroinflammation in ♂EFAD mice

Neuroinflammation is an important component of AD pathology and is modulated by *APOE* (105, 106). For example, with *APOE4* there is evidence of greater glial activation (107), higher levels of pro-inflammatory cytokines and lower levels of anti-inflammatory cytokines in humans (108-110) and in *APOE*-TR mice, both at basal levels (108, 111) and in response to an inflammatory insult (25, 106, 112-

114). In E4FAD mice, there is greater astrogliosis and microgliosis compared to E3FAD and E2FAD, particularly in the SB and deep layers of the frontal CX, brain regions with high levels of extracellular Aβ deposition, and a higher density of microglia associated with amyloid plaques (Figure 3C) (72). Further, there are higher levels of the proinflammatory cytokine IL-1β in E4FAD mice compared to E3FAD. Neuroinflammation is a complex response, involving multiple receptors and mediators, which have been assessed in EFAD mice via mRNA (106), including the complement receptor 1 (115), triggering receptor expressed on myeloid cells 2 (TREM2) (116, 117) and CD33 (118-120), all AD-relevant receptors. Further, at 6M E4FAD mice have lower levels of interleukin-4 receptor (IL-4R)-related cytokines and higher levels of toll-like receptor 4 (TLR4)-related cytokines compared to E3FAD mice (106), consistent with studies suggesting an association of IL-4R with an anti-inflammatory/repair response (121) and TLR4 with neuronal loss (122-124). Thus, E4FAD mice exhibit a neuroinflammatory phenotype which may be directly relevant to the human condition.

Tau pathology in ♂EFAD mice

In AD brains, there are higher levels of phosphorylated (p)-tau and NFT, the latter correlated with the severity of cognitive impairment (4, 29, 125-134). Although a general limitation is that FAD mice do not develop overt tau pathology, they do enable the assessment of subtler changes in phosphorylation. There are higher pTau levels in the SB and CA2/3 regions of the hippocampus (HP) in E4FAD mice at 7M (Figure 3D) not detectable at 3M (75). Thus, data from EFAD mice demonstrate that *APOE4* and Aβ interact to induce tau phosphorylation.

Neuronal viability (synaptic proteins) in ♀EFAD mice

Decreases in presynaptic and postsynaptic proteins in HP and CX contribute to altered functional connectivity, suggesting that synaptic deficits may precede frank neuronal loss in humans (135-139), eventually resulting in the cognitive impairments characteristic of AD. PSD95 and drebrin are

postsynaptic intracellular scaffold proteins commonly used to assess the structural integrity of synapses. In particular, AD patients exhibit decreased levels PSD95 and drebrin (140-144). PSD95 regulates synaptic strength and plasticity (145-149) via molecular organization of the postsynaptic region (149-151). Drebrin, a spine-resident side-binding protein of filamentous actin, regulates spine morphology, size, density and maturation (152-157) via regulation of actin cytoskeleton dynamics (158-165). In a recent publication using only ♀EFAD mice, postsynaptic proteins (PSD95 and drebrin) were reduced in the E4FAD-HP from 2-6M compared to E3FAD and E2FAD mice (Figure 4) (70). These deficits in synaptic proteins are consistent with the behavioral deficits.

Solubility of apoE and Aβ42 in ♂EFAD mice

In brain homogenates, total Aβ42 levels are 5xFAD >> E4FAD > E3FAD = E2FAD (Figure 5A) (66). To measure the solubility of both Aβ and apoE, particularly the apoE-lipoproteins in brain tissue, we adapted a 3-step sequential protein extraction protocol using TBS, TBS + Triton X-100 (TBSX), and formic acid (FA), producing extraction profiles for Aβ and apoE (Figure 5B,D) (66, 67). In the Aβ extraction profile, the 5xFAD >> E4FAD > E3FAD = E2FAD pattern was observed in the TBS and FA extraction fractions, while the TBSX fraction contains low levels of Aβ42 that are comparable across the Tg-mouse strains (Figure 5B, adapted from (66, 67)). These biochemical results are consistent with the delay in Aβ deposition induced by the replacement of m-*APOE* with h-*APOE* (Figure 3A), as are the genotype effects in levels of total and soluble Aβ42 with E4FAD > E3FAD = E2FAD (Figure 5A,B).

The apoE extraction profile detects apoE-lipoproteins in the TBSX fraction, a non-ionic detergent that releases apoE from lipoproteins without inducing the formation of new micelles, as can occur with SDS and other ionic detergents (66, 67)**.** Thus, although total levels of apoE4 < apoE3 = apoE2 in the brains of humans (103, 166, 167), *APOE*-TR mice (66, 70, 166, 168-170), and EFAD mice (Figure 5C), this isoform-specific difference is only in the TBSX-apoE4 (Figure 5D) (66). C57BL/6 Wild type (WT) mice

do not express Aβ42 but do express m-apoE and so are included for comparison with the 5xFAD mice. For total apoE, the order is the reverse of the total Aβ levels: WT < 5xFAD ≤ E4FAD < E3FAD = E2FAD (Figure 5D, adapted from (66, 67)). However, in the extraction fractions, m-apoE from the WT is detected in the TBSX fraction, while the m-apoE from the 5xFAD mice is not detectable, though low levels of m-apoE from both WT and 5xFAD are detected in the TBS and FA fractions. Thus, the presence of Aβ42 affects m-apoE solubility, specifically eliminating TBSX-m-apoE.

In the soluble fraction, the levels of the apoE isoforms are not significantly different, while soluble Aβ42 and oAβ are significantly higher with apoE4, and apoE4/Aβ complex levels are significantly lower (Figure 5E) (20, 66). Indeed, *APOE4* is characterized by increased levels of soluble Aβ (Aβ42 and oAβ) (20, 66). Soluble Aβ correlates with cognitive decline and disease severity in humans, and memory decline in FAD-Tg mice (for review (14)). To address the pathways that may underlie this correlation between apoE4 and increased soluble Aβ required the development of novel reagents, including a new mAb specific to Aβ (MOAB-2) (171) that enabled development of the critical oAβ (66) and apoE/Aβ ELISAs (20). Importantly, lower levels of apoE lipidation and apoE/Aβ complex in *APOE4* vs. *APOE3* negatively correlate with soluble Aβ levels (Figure 5E) (20, 66, 172).

Lipidomic analysis in ♂EFAD mice

Recent improvements in lipidomic technology have advanced the field to a point where it is now possible to accurately identify and quantify thousands of phospholipids (PL) in complex biological samples with relative ease. Recently, a lipidomics study revealed that the ratios of arachidonic acid (AA) and docosahexaenoic (DHA) increased in several major PL classes in the blood from cognitively normal ε4 carriers who converted to mild cognitive impairment (MCI)/AD within 3 years (173). Longitudinal profiling of E3FAD and E4FAD mice showed that blood AA- and DHA-containing PL species were altered as early as 2.5M of age. At 6M, AA- and DHA-containing lysophosphatidylcholine (LPC) species

increased in blood but decreased in the brains of E4FAD compared to E3FAD mice (173). Previous studies have shown that LPC-DHA is a preferred DHA carrier to the brain (174), and that the major facilitator superfamily domain-containing protein 2 (mfsd2a) transports this lipid across the blood-brain barrier (BBB) (175, 176). Hence, an increase in this lipid in the blood and a decrease in the brain suggests reduced transport of DHA to the brain in E4FAD mice compared E3FAD and E2FAD mice. In addition, brain DHA transport is reduced in *APOE4*-TR compared to *APOE2*-TR mice, evidence for DHA transport deficiencies (177). Collectively, these studies suggest that an imbalance in AA and DHA may be due to transport deficiencies among the ε4 carriers, which further contribute to the neuroinflammation associated with AD pathogenesis.

EFAD mice as a model for cerebrovascular dysfunction: a detailed example

CVD is re-emerging as a key component of AD, with unresolved questions as to the extent of CVD and its significance to cognitive decline. Additional issues include the apparent differences between CVD outcomes in humans and mouse models caused by *APOE4*, Aβ and sex. EFAD mice provide critical insight on the interactive role of *APOE*, ♀ sex and Aβ in CVD.

Cerebrovascular leakiness and vessel coverage in ♂ and ♀ EFAD mice

Plasma protein extravasation into the brain is a key outcome of cerebrovascular dysfunction, particularly BBB capillary leakage. Here, we demonstrate that fibrinogen levels in the CX and SB in 8M EFAD mice follow the order: ♀E4FAD > ♀E3FAD = ♂E4FAD > ♂E3FAD (Figure 6A). Total vessel coverage is a complimentary outcome measure of BBB damage, and in the SB and deep CX layers follow the order: ♂E3FAD > ♂E4FAD ≥ ♀E3FAD > ♀E4FAD (Figure 6B,C reproduced from (71)). Thus, the combination of ♀ sex, *APOE4* and Aβ induce pronounced BBB deficits that likely contribute to cognitive deficits. Indeed, high fibrinogen levels have been demonstrated in AD patients that are ε4 carriers (178) and induce glial activation and neuronal dysfunction. Angiogenic growth factors are important for

maintaining BBB function. Recent data demonstrate that plasma levels of one angiogenic growth factor, the epidermal growth factor (EGF), follows the order: ♂E3FAD > ♂E4FAD ≥ ♀E3FAD > ♀E4FAD (71). Importantly, peripheral EGF administration to mice prevented cognitive decline, cerebrovascular leakiness and vessel coverage deficits in ♀E4FAD (71). Therefore, disruption of plasma angiogenic growth factor levels is a potential downstream pathway that contributes to BBB dysfunction in EFAD mice.

Cerebral Amyloid Angiopathy (CAA) and microbleeds in ♂ and ♀ EFAD mice

CAA and microbleeds are often described as linked pathology. Here we present data that CAA in the CX is higher in E4FAD mice regardless of sex (upper layers > deep layers) but not the SB **(**Figure 7A), consistent with other investigators (73). The CAA is present primarily in larger vessels, although some capillary CAA is observed. When assessed using triple staining confocal analysis of the larger vessels, Aβ is found attached to the outside of laminin, in the perivascular space between brain endothelial cells and laminin, and penetrating the vessel (**Figure 7B**). These data raise the important general question of how Aβ in the brain interstitial fluid deposits as CAA? Identified ISF fluid drainage pathways include: 1. Perivascular flow along the capillaries to the arteriole/artery basement membrane and 2. The glymphatic pathway, in which CSF enters the brain along paravascular channels surrounding small arteries, exchanges with ISF, which is then cleared along paravascular spaces of large veins. We propose that our data are consistent with apoE4-induced impaired perivascular Aβ drainage in arterioles (179, 180), which also results in capillary CAA. In human AD patients, CAA is higher with *APOE4* (181). However, in *APOE* genotype-matched AD patients, CAA is higher in ♂ compared to ♀. While one suggestion for this apparent difference between the EFAD mice and humans is that CVD in humans is unique (73), there are several alternative explanations. First, the ♂/♀ differences in CAA may become more apparent in aged EFAD mice. Second, apoE3 may be more protective in ♀ vs. ♂ ε3/4 carriers. Third, and our hypothesis,

is that peripheral AD-risk factors are greater in ♂, inducing cerebrovascular and BBB dysfunction, leading to CAA.

In EFAD mice, microbleeds partially mimic fibrinogen extravasation, rather than CAA (71, 73, 182): ♀E4FAD > ♀E3FAD > ♂E4FAD = ♂E3FAD **(**Figure 7C (71)). In AD, microbleeds are associated with ♂, higher blood pressure, lower CSF Aβ42, and *APOE4* (183). The same considerations for CAA may underlie the microbleed differences between EFAD mice and humans. In addition, microbleeds are not severe, even in ♀E4FAD mice, and in our experience, are localized to the deeper layers of the CX. Therefore, at 8M, microbleeds could be driven by capillary or post-capillary venule breakdown, rather than CAA-induced arteriole damage. Alternatively, *APOE* modulated damage to different parts of the vascular tree may be *APOE* genotype and sex-specific, eventually leading to microbleeds.

EFAD Mice and Therapeutic Treatment

EFAD mice are a vital tool for testing therapeutics

EFAD mice exhibit temporally-defined, *APOE*-modulated changes in outcomes for efficacy (behavior, neuronal protein levels), pharmacodynamic activity (Aβ levels, neuroinflammation, and CVD) and indirect targeted engagement. Thus, the activity of therapies/drug-like molecules can be assessed in prevention, treatment and reversal paradigms. Therapies can target proximal (e.g. apoE lipidation) or downstream processes (e.g neuroinflammation) that are disrupted by *APOE4* and the detrimental interaction of ♀, *APOE4* and Aβ (e.g. sex hormone based). Further, whether a therapy is specific for *APOE4*, Aβ, or is applicable for all groups (*APOE* genotype, sex, +/-Aβ) can be determined by incorporating E2FAD, E3FAD mice, +/- FAD mutations (EFAD, EFAD-NC)). Thus far, pharmacological and non-pharmacological therapies targeting apoE-lipidation, general neuroprotection, CVD and sex hormone pathways have been tested in EFAD mice.

Targeting apoE4 lipidation

Lower lipidation/lipoprotein-associated levels in apoE4 was targeted in ♂E4FAD mice using retinoid X receptor (RXR) agonists. The history of RXR agonists in the context of AD has been extensively reviewed elsewhere (184-192). Briefly, key issues center on whether RXR agonists increase apoE levels or lipidation (via increasing ABCA1 levels), the effect of human apoE4 and the duration of treatment. In ♂E4FAD mice, short-term RXR agonist treatment (5.75-6M) increased ABCA1 levels, apoE4 lipoprotein-association/lipidation, and apoE4/Aβ complex, decreased soluble Aβ, and increased PSD95 in the HP (172). However, RXR agonists induced no beneficial effects in ♂E4FAD using a prevention protocol (5-6M) and actually increased soluble Aβ levels in ♂E3FAD and ♂E4FAD CX with the short-term protocol, possibly the result of systemic hepatomegaly. These data support RXR agonists to address the loss-of-function associated with *APOE4* and exacerbated by Aβ pathology, i.e. low levels of apoE4 lipoprotein association/lipidation. However, further development is needed to address detrimental systemic effects (RXR response elements in critical hepatic enzymes) and to discern whether RXR agonists are beneficial in specific paradigms and EFAD groups (as discussed above).

Neuroprotectants

The experimental drug NMZ was designed based on the anticonvulsant drug zendra to activate the NO/cGMP/pCREB pathway as a multifunctional protective drug. NMZ treatment of ♂E4FAD mice (3.5-6M) lowered Aβ levels (soluble and insoluble) and increased both pCREB and PSD95 (193).

Non-pharmacological treatments

Additional non-pharmacological treatments tested in EFAD mice include EGF targeting cerebrovascular dysfunction (71) and 17-β estradiol (E_2) treatment of ovariectomized (OVX) ♀EFAD mice. E_2 decreased soluble Aβ42 levels in ♀E3FAD and ♀E4FAD mice. However, insoluble Aβ levels increased in ♀E4FAD (194). Therefore, the activity of E_2 may be dependent on the relative impact of extracellular and

soluble Aβ on AD-induced neurodegeneration, with the results consistent with the hypothesis that soluble oAβ is toxic while amyloid plaques are relatively benign (Figure 1).

Potential disadvantages of the EFAD mouse model

EFAD mice share weaknesses common to all FAD-Tg mice, including questions regarding the relevance of FAD transgene-induced pathology to sporadic AD, particularly during aging. The comparison of rodent to human aging is also a construct with inherent limitations based on differences in species, and strain differences among mice. Thus, it is useful to evaluate whether FAD-Tg mice can mimic aspects of aging and AD pathology. A major issue with h-APP-Tg mice is that their 2-year life-span may not be sufficient to observe the development of AD pathology (195). As with most FAD-Tg models, AD-related pathology, particularly Aβ deposition, develops prior to middle age, which does not model the human condition. These concerns are mitigated to some extent in the EFAD by two factors. First, based on the genetic background of EFAD mice [(B6SJLF1xC57BL/6) from 5xFAD (50) X (C57BL/6) from *APOE*-TR (32)], we estimate that 10-14M will represent middle age and 18M will represent old age[g] (196). Specifically, with the known survival rates for the background strains of the EFAD mice: 1) 5xFAD have a ~75% survival rate at 16M (197): 2) C57BL/6 and *APOE*-TR have 75% survival rate at 24M for ♂ and 22M for ♀ (196). Thus, the EFAD mice have ~75% survival at 20M for ♂ and 19M for ♀, making our target "old age" 18M. Although specific measures of AD pathology in the EFAD are significant by 6M, pathology continues to develop until at least 18M, the oldest EFAD mice we have examined thus far. Second, the EFAD-NC littermates provide both a comparison to the EFAD mice and a complementary approach to the address functional questions about *APOE* in the absence of FAD-induced pathology.

Despite these limitations, **EFAD mice are the only well-characterized FAD/h-*APOE*-Tg mouse model with an extensive and growing provenance.** Consistent with human AD patients, E4FAD mice develop pathology in a number of *APOE* genotype-, sex-, and age-dependent pathways. EFAD mice are a tractable mouse model to study a number of AD-related outcomes, including changes in behavior, Aβ deposition,

tau pathology, neuroinflammation and neuronal viability (Table 2), as well as apoE lipidation and Aβ solubility (Table 3). These mice also allow for study of the interactions among AD risk factors, including age, *APOE* genotype and sex.

Future Directions

Using EFAD mice as a model of aging and development of AD pathology

Understanding the interaction and dominance of APOE genotype vs. sex with aging

Identification of the interactions between *APOE* genotype and sex are critical to understanding both aging and the development of AD pathology. Making predictions requires identification of the dominant risk factor in a given comparison, *APOE* genotype or sex (1). The levels of Aβ and amyloid deposition, as well as soluble Aβ levels are higher in ♀ vs. ♂ in several FAD-Tg mice (Tg2576, APP/PS1, 3xTg-AD) (64, 73, 198-203), as well as the EFAD mice (Table 3) (2). In *APOE*-Tg mice, cognitive deficits are greater in ♀ *APOE4* vs. *APOE3* (for review (21), (204-209)). In EFAD mice, behavioral deficits are E4FAD > E3FAD and ♀>♂ in 6M and 8M mice (Figure 2, Table 2) (3). In humans, lifetime AD risk, cognitive decline and accumulation of Aβ is ♀>♂ in ε4 carriers. These data suggest that the greatest risk for AD is with ♀ *APOE4* carriers (6-12, 102, 210-213). These observations introduce a reoccurring theme in this field of research: which risk factor is dominant in its effects on AD pathology: *APOE* genotype or sex, and does this change with age? Based on cognition and AD-related histopathology, our general predicted order for AD pathology, with the addition of heterozygous E3/4FAD, is: **♂E3FAD < ♀E3FAD < ♂E3/4FAD < ♀E3/4FAD < ♂E4FAD < ♀E4FAD** (the effect of the *APOE2* genotypes are discussed separately). However, the dominant risk factor in a given comparison, *APOE* genotype or sex, is unclear. In general, the key comparisons for establishing the dominant effects of *APOE* vs. sex will be determined by heterozygous E3/4FAD mice vs. homozygous E3FAD and E4FAD mice, as established by age and AD pathology. For example, Aβ deposition, neuroinflammation and tau pathology in ♂E4FAD vs. ♀E3/4FAD will predict a dominant risk factor: ***APOE4*** if ♂E4FAD shows the greatest pathology, or

♀sex if ♀E3/4FAD has the greater pathology. How this relative risk changes with age is critical. As well, using the EFAD-NC we can determine the effect of *APOE* vs. sex interactions on normal aging.

Understanding trajectories, cliffs and therapeutic windows

Multiple measures of AD pathology during aging will inform two critical components that indicate the relative contribution of risk factors, *APOE* or sex, and how their contributions are altered along the trajectory of the disease. 1) "Cliffs" or tipping points suggest a clear dominant risk factor: ♀ sex or *APOE*. For example, while ♀E4FAD mice exhibit the greatest behavioral deficits and Aβ pathology at both 6M and 8M, ♀E3FAD ≈ ♂E4FAD at 8M (71), suggesting a "cliff" or tipping point where ♀ sex is dominant compared to *APOE* genotype. However, unlike humans, as the ♀EFAD mice age, they maintain 45-80% E_2 levels and normal uterine weight (214-218), which may produce an interesting phenotype at older ages with the scale tipping towards the dominance of *APOE* genotype. This change can be compared to OVX +/- E_2 replacement. 2) Therapeutic windows are periods during which specific components of AD pathology are differentially affected by *APOE* or ♀ sex, allowing us to design and test specific therapeutic targets in preclinical studies using prevention or reversal paradigms.

Understanding the function of APOE2

The majority of the published data on the EFAD mice have used ♂ mice ≤8M. As ♂ and ♀mice are aged from 10M-14M-18M, sex and *APOE* genotype interact to induce significant differences in various components of AD pathology (preliminary data). As well, all our work thus far has been with *APOE* $^{+/+}$/5xFAD$^{+/-}$ mice. As the *APOE* heterozygous genotypes are investigated (ε2/3, ε2/4, ε3/4), the influence of *APOE* genotype and sex interactions can be fully defined. In studying the *APOE2* genotypes, it is important to keep in mind that if there are functional differences among ε2/2, ε2/3 and ε2/4, it will likely go unidentified in all but the largest human cohort studies. This is because most studies will be underpowered for significance because of the low frequency of the ε2 alleles (estimated: ε2/2 at 0.4%,

ε2/3 at 8.8% and ε2/4 at 1.5%; (5, 6)). This effect is exacerbated if the *APOE2* genotypes are further stratified by age, AD status and sex, resulting in the apparently contradictory literature for this field. However, heterozygous genotypes of *APOE2* mice can be bred to reach significance via power analysis for any variable in comparison to heterozygous genotypes of *APOE3* and *APOE4.* Indeed, the study of ε2/2, ε2/3 and ε2/4 is perhaps a more subtle model to study the protective the effects in both a normal (EFAD-NC) and AD (EFAD) cohort of mice. These results are key for identifying how the genotypes of *APOE2* may cause differential effects in the context of being protective factors, for example, does ε2/4 behave more like the risk ε4 or the protective ε2. These studies will provide new insights into how *APOE2* imparts healthy brain aging and reduces AD risk, leading to diagnostic biomarkers and identification of therapeutic targets.

Using EFAD to identify environmental risk factors in AD pathology

About 98% of the human AD cases are sporadic with only half the cases linked to *APOE4* and other genetic loci identified by GWAS, suggesting the presence of other generic or environmental risk factors and thus the potential interaction between genetic and environmental risk factors (88, 219-227). Thus, while *APOE4* is the major genetic risk factor for AD, a number of environmental or lifestyle risk factors, have also been identified (228-233). Two examples are given below,

Effect of high fat diets on AD

Epidemiological studies in humans consistently show an interaction between obesity and dementia/increased AD risk (228, 234-239), though the interaction with sex remains controversial sex (240-243). High fat diet-induced obesity accelerates AD pathology in FAD-Tg mice (244-248) and impairs cognition in *APOE4*-TR mice (249). However, the interaction among obesity, *APOE* genotype and sex in modulating development of AD pathology is poorly understood (250, 251). EFAD mice are a

relevant model to address this question and the importance of lifestyle risk factors and their association with *APOE* in a genotype- and sex-dependent manner.

Effect of particulate air pollutants

The role of particulate air pollutants in accelerating cognitive impairment has been established in human (252-255) and WT mouse studies (256). Exposure to particulate air pollutants increased Aβ deposition, amyloid plaques and soluble oAβ in ♀E4FAD compared to ♀E3FAD mice (257). This increased susceptibility of ♀ε4 carriers to the neurotoxicity of particulate air pollutants provides evidence for interactive effects among genetic and environmental risk factors.

Using EFAD as a therapeutic model

Repurposing cardiovascular disease drugs

As discussed above, we previously demonstrated that in EFAD mice, induction of ABCA1/ABCG1 with RXR agonists increased apoE4 lipoprotein-association/lipidation, decreased soluble Aβ, and increased PSD95 in the HP (172). However, treatment induced severe hepatomegaly, limiting RXR agonism for AD treatment. Approaches for targeting apoE lipoprotein-association/lipidation in brain without the use of RXR agonists emerged as a promising alternative as the major enzymatic and lipid transport activities involved in the peripheral system are also expressed in the brain (258-279). The lipoprotein-association/lipidation of apoE in the brain parenchyma is the result of intercellular lipoprotein maturation and remodeling (263, 274, 280-291). Current strategies include directly targeting ABCA1 activity with apoE mimetic peptide are being evaluated in the EFAD mice as a therapy for increasing apoE levels or apoE4 lipoprotein-association/lipidation and reducing toxic soluble Aβ peptides, resulting in neuroprotection against AD pathology.

Cerebrovascular dysfunction (CVD)

Many of the planned treatment strategies that target either the proximal or downstream processes modulated by *APOE* and sex will likely also target CVD. Proximally, directly targeting the structural and functional deficits of apoE4 may ameliorate detrimental changes that cause CVD (190, 292-294). Targeting downstream signaling pathways or the soluble mediators produced by *APOE*-modulated activated glia (astrocytes and microglia) and pericytes may ameliorate CVD, or prevent the risk with a subsequent additional hit such as peripheral inflammation and high fat diets (292). Further, brain endothelial cells are often overlooked as a direct therapeutic target. The advantages of this target include: 1. Brain penetration is not required; 2. Peripheral risk factors will likely initially target brain endothelial cells rather than cells in the brain and; 3. As highlighted by the EGF treatment study, as brain endothelial cells play a central role in the homeostasis of the CNS, targeting brain endothelial cells may induce a pronounced beneficial effect on cognition. Currently, the ability of EGF to reverse cognitive and cerebrovascular deficits is under evaluation.

Neuroinflammation

Epidemiological studies targeting peripheral inflammation for AD indicate *APOE*-dependent lowering of AD risk due to nonsteroidal anti-inflammatory drugs (NSAIDs), with a beneficial effect for ε4 resulting in initiation of AD Anti-inflammatory Prevention Trial (ADAPT) (295-304). However, ADAPT failed and led to more criticism for evaluating the role of neuroinflammation in AD. It remains unclear whether targeting AD-relevant neuroinflammation receptor pathways is beneficial, detrimental or not effective. For example, data from FAD-Tg mice provide evidence for beneficial (25, 305-307) and detrimental effects from TLR4 inhibition (308-311). Inflammatory receptors may function differently depending on stage of AD pathology and *APOE* genotype, necessitating prevention and treatment protocols. EFAD mice are an ideal model to investigate this interplay between neuroinflammation and neurodegeneration that result in cognitive behavioral impairments, and for identifying the appropriate timing and targets involved in AD-associated neuroinflammation. Currently, EFAD are being evaluated with a prevention and reversal paradigm trial with a small TLR4 antagonist to evaluate its effect on AD pathology.

SEMs and SERMS

E_2 is key for ♀ vulnerability to *APOE4*-induced AD risk and pathology: OVX-induced loss of circulating E_2 in pre-menopausal women (312-316) and FAD-Tg mice (201, 317, 318) causes cognitive deficits that can be reversed by E_2 and estrogen therapy (ET), and in FAD-Tg mice, the OVX-induced increase in amyloid deposition is also reversed with ET (319, 320). However, the timing of ET in relation to the risk of AD in naturally menopausal women is a critical factor due to the apparent opposing outcomes based on early vs. late menopause treatment (321). The controversial outcomes associated with timing could be addressed with the development of safe ET alternatives (Alt-ET) for the prevention and treatment of AD, potentially specific for the *APOE* genotype of patient. Based on the need for Alt-ET, we plan to study SERMs (selective estrogen receptor modulator) (322-325), or SEMs (selective estrogen mimic) (326) in +/- OVX ♀ EFAD mice.

Summary

Given the prevalence of AD and the repeated failure of clinical trials, it is critical to develop Tg-mouse models to understand the mechanisms driving the trajectory of AD, identify early-stage biomarkers, and test preclinical therapeutic targets. EFAD mice mimic a range of AD-related pathologies including cognitive decline, region-specific Aβ and plaque deposition, progressive neuroinflammation, reduced synaptic viability and cerebrovascular dysfunction. EFAD mice provide insight into the specific pathways and mechanisms that underlie *APOE*- and sex-dependent modulation of AD pathology. A complete characterization of the EFAD mice with age will enable an understanding of how the interaction among the greatest AD risk factors modulate AD-related pathology, specifically age, *APOE* genotype, and sex. Consistent with the underlying principles of personalized medicine, only when we understand these interactions can we begin to design therapeutic approaches for the prevention and treatment of AD.

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FOOTNOTES

a. https://endpts.com/mercks-leading-phiii-bace-drug-implodes-in-latest-alzheimers-disaster/

b. http://www.thetimes.co.uk/article/how-mice-immunity-is-hindering-research-n5fl2j5fj

c. https://www.theatlantic.com/health/archive/2017/02/alzheimers-amyloid-hypothesis/517185/

d. http://www.monsterinthemind.com/

e. https://www.youtube.com/watch?v=6tBSFEzkk0A

f. https://www.nia.nih.gov/research/dab/aged-rodent-colonies-handbook/strain-survival-information

Table 1. Effects of human *APOE* on Aβ pathology in FAD-Tg mice.

MODEL	Aβ PATHOLOGY				
	<4M	**4-8M**	**8-12M**	**12-16M**	**≥ 16M**
A. FAD-Tg		↑	↑↑	↑↑↑	↑↑↑↑
B. FAD-Tg X *APOE*-KO			↑	↑↑	↑↑↑
C. FAD-Tg X *APOE*-Tg				↑ (ε4>ε3)	↑↑ (ε4>ε3)
D. 5xFAD$^{+/-}$	↑↑	↑↑↑	↑↑↑↑	↑↑↑↑↑	↑↑↑↑↑↑
E. 5xFAD$^{+/-}$ X *APOE*-TR = EFAD-Tg		↑ (ε4>ε3)	↑↑ (ε4>ε3)	n.m	n.m

Differences in Aβ pathology (measured as Aβ levels using biochemical methods or Aβ deposition using IHC) in FAD-Tg mice in the presence or absence of m- or h-*APOE* genotypes across the mouse lifespan among **A.** FAD-Tg (27, 33-35, 37-44, 46, 327, 328)**, B.** FAD-Tg/*APOE*-KO (27, 30, 39, 40, 46), **C.** FAD-Tg/*APOE*-Tg (27, 28, 30, 47, 48), **D.** 5xFAD-Tg (50, 55, 58, 62-64, 68, 69, 222, 329-339), **E.** 5xFAD/*APOE*-TR (EFAD-Tg mice) (20, 66, 70-75, 257). Aβ pathology change: low ↑ to high ↑↑↑↑↑, relative to an earlier age or among FAD-Tg mouse models. If measured and significant, differences between sexes or *APOE* genotypes are specifically indicated within each cell (as applicable). n.m: not measured.

Table 2. AD-related behavioral deficits, histopathology and loss of neuronal viability in 5xFAD and EFAD mice.

MODEL	PHENOTYPE					
		<4M	4-8M	8-12M	12-16M	≥ 16M
5xFAD	**Behavioral deficits**					
	Learning, exploration and memory	↑ (MWM)	↑↑ (YM, MWM, FC, NOR)	↑↑↑ (YM, MWM, FC, CTA)	↑↑↑↑ (YM, MWM, FC, CM)	↑↑↑↑ (YM)
	Histopathology					
	Aβ deposition	↑↑	↑↑↑	↑↑↑↑	↑↑↑↑↑	↑↑↑↑↑↑
	Plaque deposition	↑↑	↑↑↑ (♀> ♂)	↑↑↑↑ (♀> ♂)	↑↑↑↑ (♀> ♂)	n.m.
	CAA	↑	↑↑	↑↑↑	n.m.	n.m.
	Neuroinflammation	↑	↑↑	↑↑↑	↑↑↑↑	↑↑↑↑
	pTau	↑	↑	↑↑	----	n.m.
	Neuronal viability					
	Synaptic protein loss	↑	↑↑	↑↑↑	↑↑↑	n.m.
	Neuroplasticity	↑	↑↑	↑↑↑	n.m.	n.m.
	Neuronal loss	----	↑*	↑↑*	↑↑↑	n.m.
EFAD	**Behavioral deficits**					
	Learning and exploration and memory	----	↑ (MWM/YM: ♀ε4 > ε3 ≥ ε2)	↑ (YM: ε4 > ε3; ε4 ♀> ♂, NOR: ε4 ♀>♂; ♀ε4 > ε3)	n.m.	n.m.
	Histopathology					
	Aβ deposition	----	↑ (ε4 > ε3 ≥ ε2; ♀> ♂)	↑↑ (ε4 > ε3; ♀> ♂)	n.m.	n.m.
	Plaque deposition	----	↑ (ε4 > ε3 ≥ ε2; ♀> ♂)	n.m.	n.m.	n.m.
	CAA	n.m.	↑ (ε4 > ε3; ♀= ♂)	n.m.	n.m.	n.m.
	Neuroinflammation	n.m.	↑ (ε4 > ε3)	n.m.	n.m.	n.m.
	pTau	----	↑ (ε4 > ε3)	n.m.	n.m.	n.m.
	Neuronal viability					
	Synaptic protein loss	----	↑ (♀ε4 > ε3 ≥ ε2; ♂ε4 > ε3)	n.m.	n.m.	n.m.
	Neuroplasticity	n.m.	n.m.	n.m.	n.m.	n.m.
	Neuronal loss	n.m.	n.m.	n.m.	n.m.	n.m.

TABLE 2. AD-related behavioral deficits, histopathology and loss of neuronal viability in 5xFAD and EFAD mice.

5xFAD mice: Behavioral deficits: Morris water maze (MWM) (49, 50, 59-61, 340), Y-maze (YM) (49, 51, 54, 55, 58), fear conditioning (FC) (52, 56, 57), novel object recognition (NOR) (330), conditioned taste aversion (CTA) (52), cross-maze (CM) (62); AD-related histopathology: Aβ deposition: IHC-mAb to Aβ, plaques: Thio-S, cerebral amyloid angiopathy (CAA): Thio-S, methoxy-X04 (26, 50, 52, 55, 58, 62-64, 68, 69, 329-337, 341), neuroinflammation: astrogliosis or microgliosis IHC-mAb to GFAP or Iba1/F4-80 (50, 55, 58, 61, 329, 333-335), pTau: Western blot for p-sites (59, 342); Neuronal viability: Synaptic proteins: PSD95, synaptophysin, syntaxin by Western blot or IHC (50, 68, 69), neuroplasticity: basal synaptic transmission, long-term potentiation or paired-pulse facilitation (56, 57, 61, 330, 331, 335, 336, 343-345), neuronal loss: cresyl violet, NeuN IHC, quantified by area* (50, 185), or using unbiased stereology (61-63, 68, 69, 334, 338, 346).

EFAD mice: Behavioral deficits: MWM, YM, NOR (70, 71); AD-related histopathology: Aβ deposition: IHC Aβ-specific MOAB-2 for plaques (66, 71, 73), CAA: IHC mAb and Thio-S (66, 72, 73, 257), neuroinflammation: IHC-mAb to GFAP for astrogliosis or Iba1 for microgliosis (72), pTau: Western blot for p-sites (347); Neuronal viability: synaptic proteins: PSD95, synaptophysin, drebrin by Western blot (70, 172).

The levels are represented as low ↑ to high ↑↑↑↑↑↑ relative to an earlier age or, if known and significant, differences between sex or *APOE* genotypes are specifically indicated within each cell relative to an earlier age. n.m.: not measured; ---- : not detectable.

Table 3. Differences in AD-related biochemical measures in 5xFAD and EFAD mice.

MODEL	BIOCHEMICAL MEASURES					
		<4M	**4-8M**	**8-12M**	**12-16M**	**≥ 16M**
5xFAD	**Aβ and apoE solubility**					
	Total Aβ□	↑ (♀> ♂)	↑↑ (♀> ♂)	↑↑↑ (♀> ♂)	↑↑↑↑	↑↑↑↑↑
	Soluble Aβ42	↑	↑↑	↑↑↑	n.m	n.m
	Total apoE	↑	↑↑	↑↑↑	↑↑↑↑	n.m
	TBSX-apoE	----	----	n.m	n.m	n.m
	APP processing					
	APP levels	↑	↑↑	↑↑↑	↑↑↑	↑↑↑
	BACE levels	↑	↑↑	↑↑↑	↑↑↑	↑↑↑
	C-terminal fragments	↑	↑↑	↑↑↑	↑↑↑↑	↑↑↑↑
	Neurotrophic factors					
	BDNF	↓	↓↓	n.m	↓↓↓	↓↓↓↓
	pCREB	n.m	----	↓	n.m	n.m
	Neuroinflammatory cytokines					
	TNF-α	↑	↑	↑↑	n.m	n.m
	IL-1β	↑	↑↑	n.m	n.m	n.m
EFAD	**Aβ and apoE solubility**					
	Total Aβ□	----	↑ (ε4 > ε3 ≥ ε2)	n.m	n.m	n.m
	Soluble Aβ42	----	↑ (ε4 > ε3 ≥ ε2; ε4 ♀> ♂)	n.m	n.m	n.m
	Total apoE	n.c (ε4 < ε3 ≤ ε2)	n.c (ε4 < ε3 ≤ ε2)	n.m	n.m	n.m
	TBSX-apoE	n.c (ε4 < ε3 ≤ ε2)	n.c (ε4 < ε3 ≤ ε2)	n.m	n.m	n.m
	APP processing					
	APP levels	n.m	n.c (ε4 = ε3 = ε2)	n.m	n.m	n.m
	BACE levels	n.m	n.m	n.m	n.m	n.m
	C-terminal fragments	n.m	n.m	n.m	n.m	n.m
	Neurotrophic factors					
	BDNF	↓ (♀ε4 < ε3 ≤ ε2)	↓↓ (♀ε4 < ε3 ≤ ε2)	n.m	n.m	n.m
	pCREB	↓ (♀ε4 < ε3 ≤ ε2)	↓↓ (♀ε4 < ε3 ≤ ε2)	n.m	n.m	n.m
	Neuroinflammatory cytokines					
	TNF-α	n.m	↑ (ε4 > ε3)	↑ (ε4 = ε3)	n.m	n.m
	IL-1β	n.m	↑ (ε4 > ε3)	↑ (ε4 = ε3)	n.m	n.m

TABLE 3: Differences in AD-related biochemical measures in 5xFAD and EFAD mice.

5xFAD mice: ApoE and Aβ solubility: total Aβ: by ELISA (49, 50, 52, 53, 57, 66, 222, 348), soluble Aβ42: quantified in TBS or PBS extraction fraction by ELISA (67, 334, 338, 348), total apoE: by ELISA or Western blot (67, 348, 349); TBSX-apoE: measured in TBSX extraction fraction by ELISA(67); APP processing: APP, BACE, C-terminal fragments by Western blot (49, 52, 53, 58, 67, 222, 336, 337, 339, 348-352); Neurotrophic factors: BDNF and pCREB proteins by Western blot or mRNA by qPCR (55, 61, 336, 350, 352, 353); Neuroinflammatory cytokines: TNF-α, IL-1β mRNA by qPCR (185, 342, 351, 354).

EFAD mice: ApoE and Aβ solubility: total Aβ by ELISA (66, 172), soluble Aβ42: quantified in TBS extraction fraction by ELISA (66, 73, 172), total apoE by ELISA (66, 70), TBSX-apoE: measured in TBSX extraction fraction by ELISA (66, 172); APP processing: APP by Western blot; Neurotrophic factors: BDNF and pCREB proteins by Western blot (70, 193); Neuroinflammatory cytokines: TNF-α, IL1-β mRNA by qPCR (72, 106).

Change: Direction: increase ↑, decrease ↓. Extent: the number of arrows (lower-higher) relative to an earlier age. If known and significant, differences between sex or *APOE* genotypes are specifically indicated within each cell relative to an earlier age. n.c: no-change; n.m: not measured; ---- : not detectable.

FIGURES

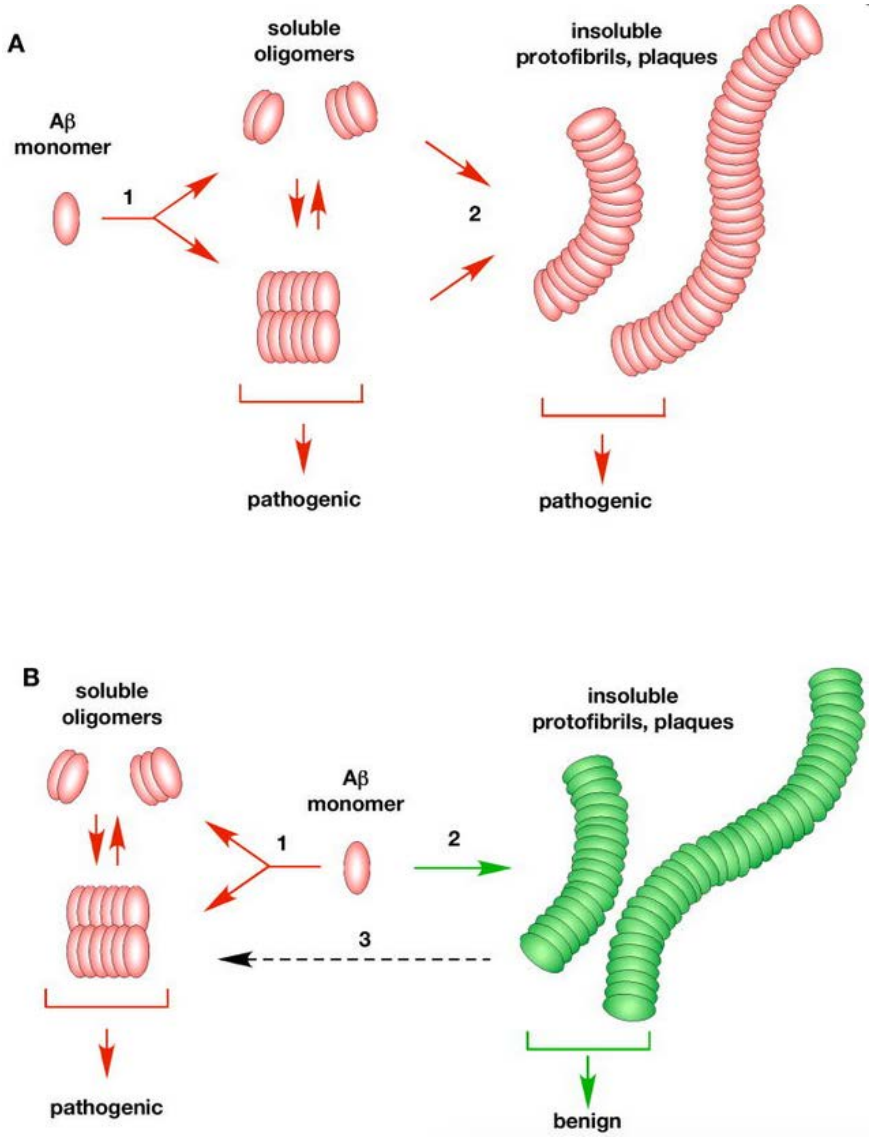

Figure 1: Aβ assembly pathways: oAβ "on" or "off" pathways for amyloid plaques deposition. A. Via the "on" pathway, oAβ can be pathogenic, but are structural precursors for the formation of the traditional insoluble fibrils that continue to assemble into the parallel β-sheet structure of amyloid plaques, also considered to be pathogenic. **B.** Via the "off" pathway assembly process, Aβ can proceed via two separate processes, the pathogenic formation of oAβ or formation of the traditional insoluble fibrils that continue to assemble into amyloid plaques, considered to be benign (reproduced from (86)).

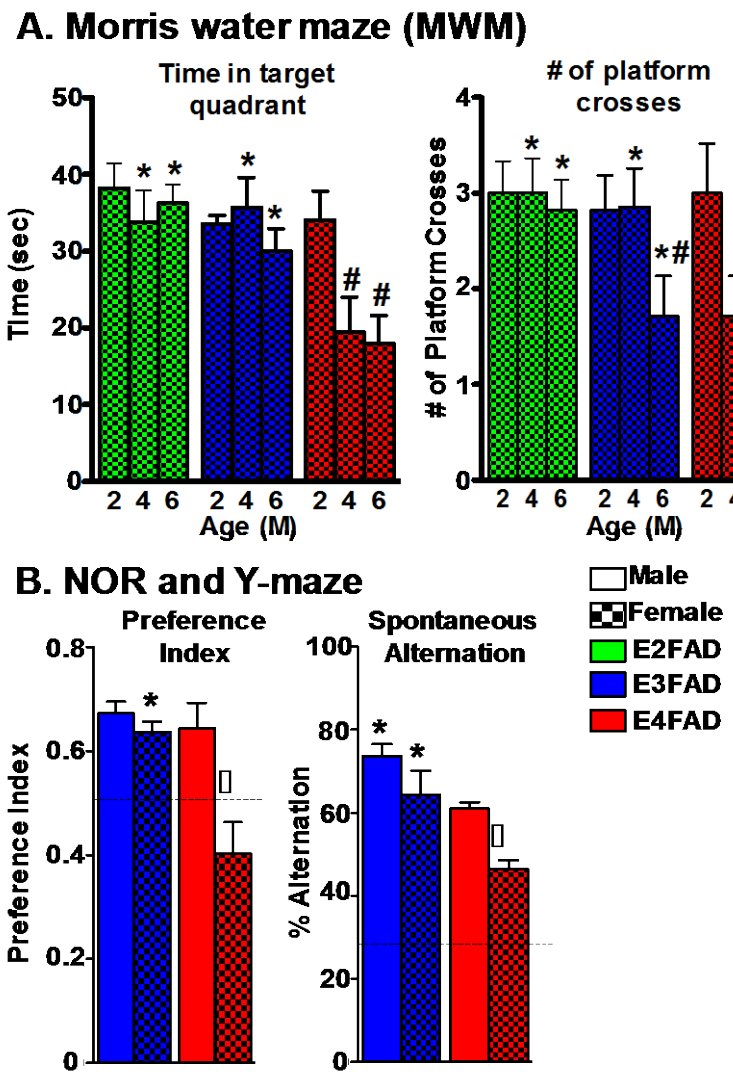

Figure 2: Behavior deficits in EFAD mice. A. MWM in 2, 4, and 6M ♀EFAD mice, behavior preformed during light cycle (adapted from (70)). **B**. NOR preference index and YM spontaneous alternation in 8M ♂ and ♀ EFAD mice, behavior preformed during dark cycle (adapted from (71)). $p<0.05$: * vs. E4FAD within sex, # vs. 2M, † ♀ vs. ♂ within genotype.

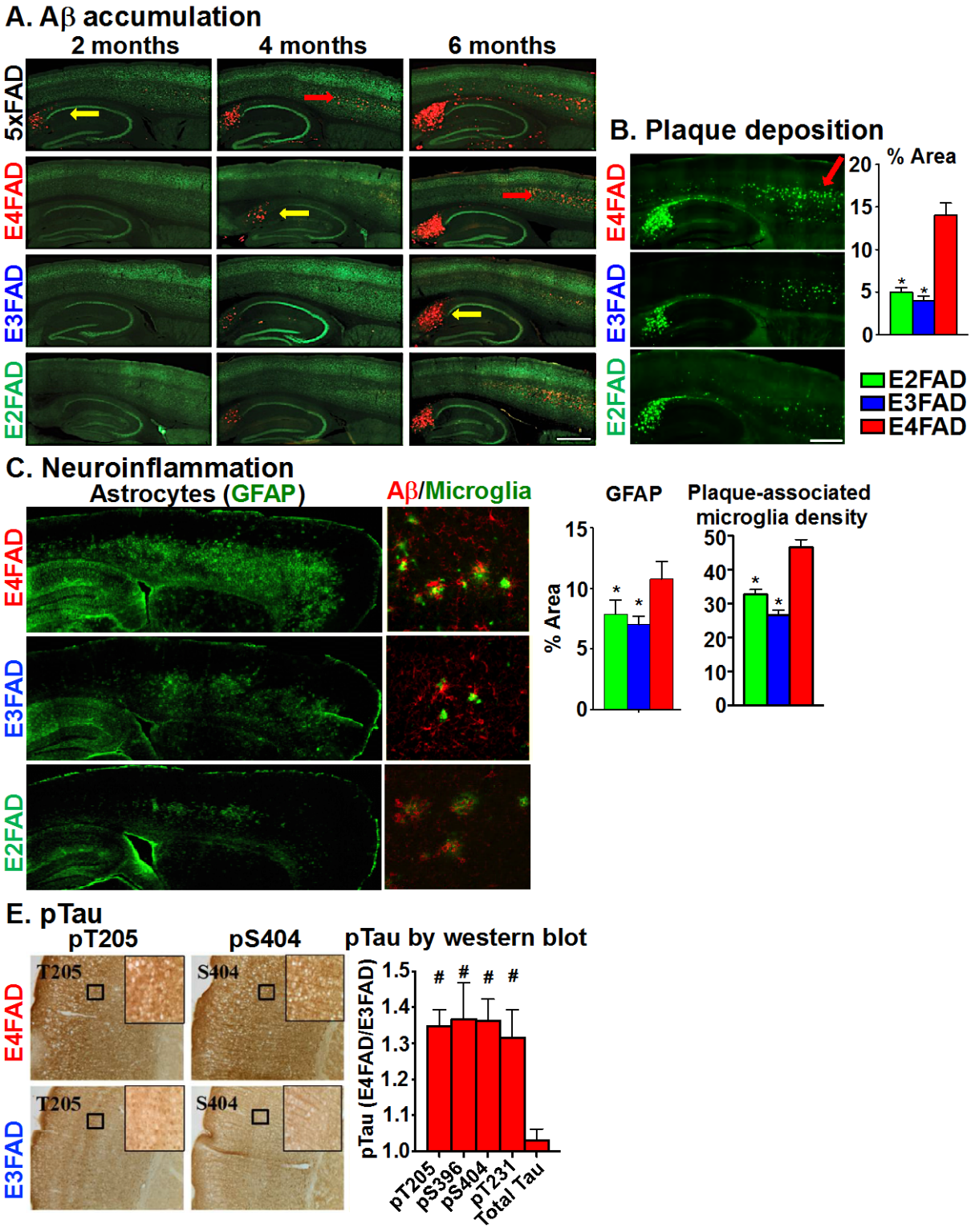

Figure 3: AD histopathology in 2-6M ♂ 5xFAD and EFAD mice. **A**. Total Aβ deposition by IHC in 5xFAD and ♂EFAD sagittal brain sections at 2, 4, 6M with mAb for Aβ (MOAB-2, red) and mAb for

neurons (NeuN, green). **B**. Plaque deposition: Thio-S staining in 6M ♂EFAD sagittal brain sections, quantified by % area of CX (reproduced from (66)), * $p<0.05$ vs. E4FAD. **C**. Neuroinflammation: IHC staining in 6M ♂EFAD sagittal brain sections for astrocytes (GFAP) (adapted from (72)), quantified as % area of CX; and for reactive microglia (Iba1, green) and Aβ (MOAB-2, red), quantified as plaque-associated microglial density (reproduced from (72)), $p<0.05$: * vs. E4FAD. **D**. pTau: IHC in 7M ♂EFAD CX for phosphorylated Tau pT205 and pS404 sites. Insets: 20X magnification (black box). pTau quantified in EFAD CX by Western blot, expressed as E4FAD/E3FAD, #$p<0.05$ vs. E3FAD (reproduced from (347)). Arrows for pathology: yellow=HP, red=CX.

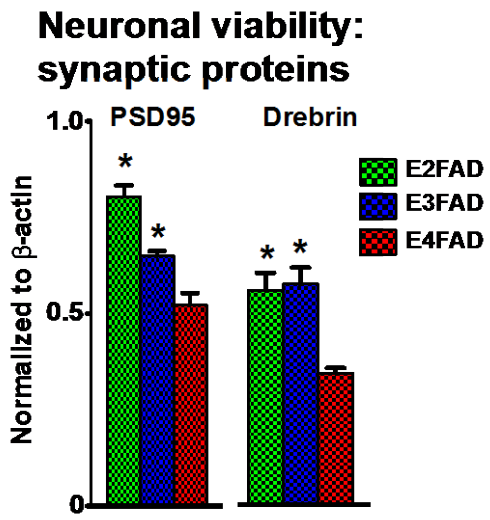

Figure 4: Synaptic protein levels in ♀EFAD mice. Synaptic proteins PSD95 and drebrin measured in 6M ♀ EFAD mice by Western blot and normalized to β-actin (adapted from (70)), * $p<0.05$ vs. E4FAD.

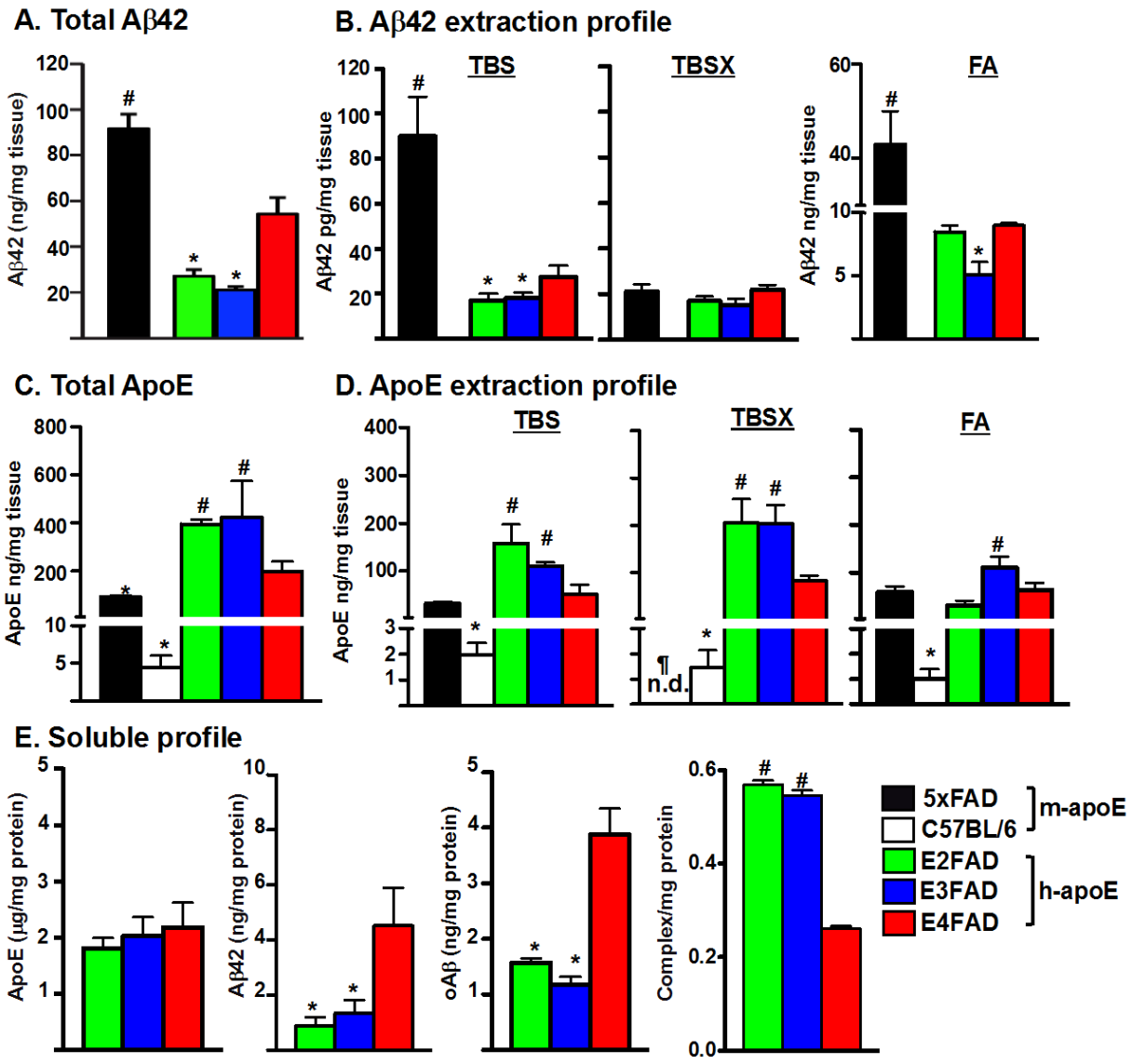

Figure 5: Aβ42 and apoE solubility in 6M ♂ 5xFAD, C57BL/6 and EFAD mice. A. Total Aβ42 levels in the brain. **B.** Aβ42 extraction profile after sequential extraction of CX with TBS, TBSX and FA (adapted from (66, 67)). **C.** Total apoE levels in the brain. **D.** ApoE extraction profile of CX, as described for B. (adapted from (66, 67)). **E.** Soluble profile (TBS fraction): apoE, Aβ42, oAβ and apoE/Aβ complex from HP of EFAD mice (adapted from (20, 66, 67)). * p< 0.05 significantly less than E4FAD, # p< 0.05 significantly greater than E4FAD, ¶ p< 0.05 significantly less than C57BL/6. All values are represented as mean ± S.E. normalized per mg tissue (A-D) or per mg protein (E).

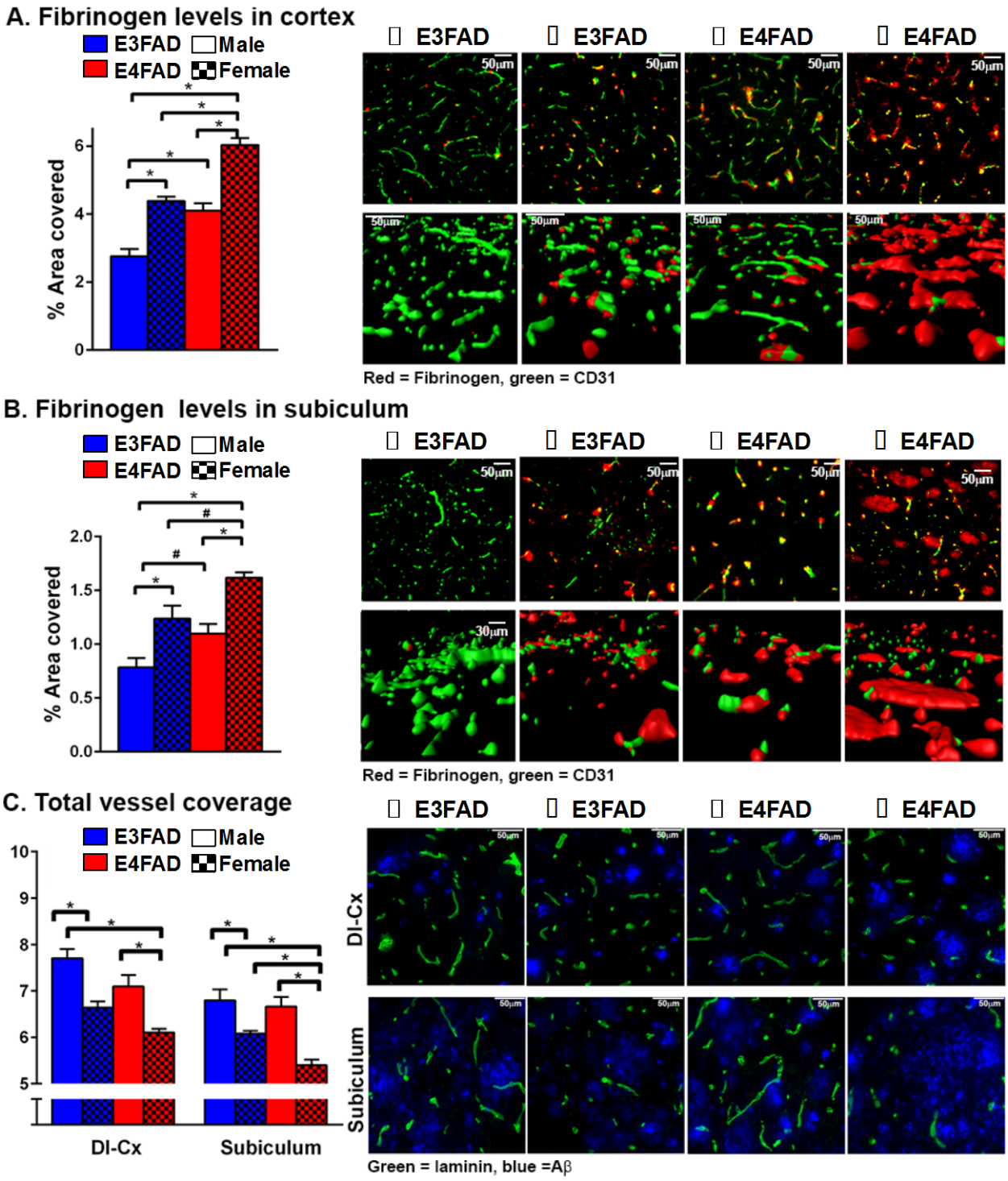

Figure 6: Cerebrovascular leakiness and vessel coverage in 8M EFAD mice. Fibrinogen (red) levels in the **A.** CX and **B.** SB follow the order: ♀E4FAD > ♀E3FAD = ♂E4FAD > ♂E3FAD. Green = CD31. *n* = 8. Data expressed as mean +/- S.E.M. **p*<0.05 by two-way ANOVA and Tukey's post hoc

comparisons. #$p<0.05$ by two-way AVOVA followed Fisher's LSD test (new data). **C.** Laminin (green) staining as a marker of total vessel coverage follows the order: ♂E3FAD > ♂E4FAD ≥ ♀E3FAD > ♀E4FAD in the deep CX and SB (adapted from (71)).

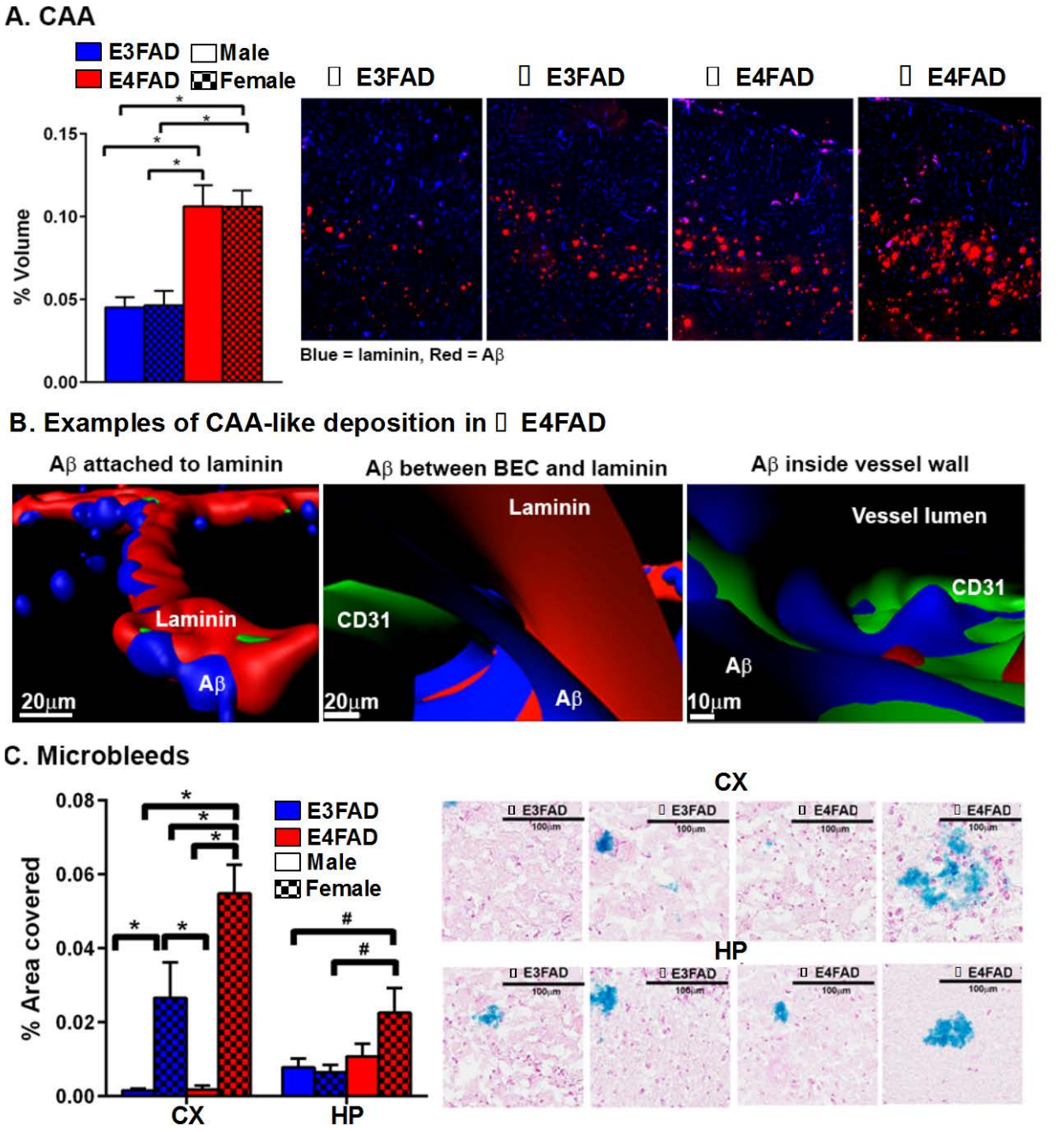

Figure 7: CAA and microbleeds in 8M EFAD mice. A. Cortical CAA-like deposition is highest in ♀E4FAD and ♂E4FAD. *n* = 8. Data expressed as mean +/- S.E.M. **p*<0.05 by two-way ANOVA and Tukey's post hoc comparisons (new data). **B.** Aβ deposits are found attached to laminin, in the perivascular space and inside the vessel lumen. Examples from ♀E4FAD mice (new data). **C.** Microbleeds are highest in ♀E4FAD mice (adapted from (71)).