



Thematic Review Series: ApoE and Lipid Homeostasis in Alzheimer's Disease

EFAD transgenic mice as a human APOE relevant preclinical model of Alzheimer's disease^SLeon M. Tai,* Deebika Balu,* Evangelina Avila-Munoz,* Laila Abdullah,[†] Riya Thomas,* Nicole Collins,* Ana Carolina Valencia-Olvera,* and Mary Jo LaDu^{1,*}Department of Anatomy and Cell Biology,* University of Illinois at Chicago, Chicago, IL 60612; and Roskamp Institute,[†] Sarasota, FL 34234

Abstract Identified in 1993, *APOE4* is the greatest genetic risk factor for sporadic Alzheimer's disease (AD), increasing risk up to 15-fold compared with *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 affect AD pathology has been limited by the lack of a tractable familial AD-transgenic (FAD-Tg) mouse model expressing h-*APOE* rather than mouse (m)-*APOE*. The disparity between m- and h-apoE is relevant for virtually every AD-relevant pathway, including amyloid- β (A β) 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.^S Here we review insights gleaned from the EFAD mice and summarize future directions.—Tai, L. M., D. Balu, E. Avila-Munoz, L. Abdullah, R. Thomas, N. Collins, A. C. Valencia-Olvera, and M. J. LaDu. **EFAD transgenic mice as a human *APOE* relevant preclinical model of Alzheimer's disease.** *J. Lipid Res.* 2017. 58: 1733–1755.

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INTRODUCTION

Alzheimer's disease: 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 with the common *APOE3* genotype, while *APOE2* is protective (3, 4), but less frequent [estimated: $\epsilon 2/2$ at 0.4%, $\epsilon 2/3$ at 8.8%, and $\epsilon 2/4$ 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

Abbreviations: AA, arachidonic acid; A β , amyloid- β ; AD, Alzheimer's disease; APP, amyloid precursor protein; BBB, blood-brain barrier; CAA, cerebral amyloid angiopathy; CSF, cerebrospinal fluid; CerVD, cerebrovascular dysfunction; CX, cortex; E₂, 17- β estradiol; EFAD-NC, EFAD noncarrier; EGF, epidermal growth factor; ET, estrogen therapy; FAD, familial Alzheimer's disease; GFAP, glial fibrillary acidic protein; h-*APOE*, human *APOE*; HP, hippocampus; IL-1 β , interleukin-1 β ; IL-4R, interleukin-4 receptor; mAb, monoclonal antibody; m-*APOE*, mouse *APOE*; MOAB-2, mAb specific to amyloid- β ; MWM, Morris water maze; NFT, neurofibrillary tangle; NOR, novel object recognition; NSAID, nonsteroidal anti-inflammatory drug; oA β , oligomeric conformation of amyloid- β ; OVX, ovariectomized; pCREB, phosphorylated cAMP-response element binding protein; PL, phospholipid; PSD95, postsynaptic density protein 95; pTau, phosphorylated tau; RXR, retinoid X receptor; SB, subiculum; TBSX, TBS + Triton X-100; Tg, transgenic; Thio-S, thioflavine-S; Thy-1, thymocyte differentiation antigen 1 theta; TLR4, toll-like receptor 4; TR, targeted-replacement; YM, Y-maze; ♀ , female; ♂ , male.

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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 (NFTs) of tau. In addition, soluble oligomeric conformations of amyloid- β ($\text{oA}\beta$) correlate with cognitive decline and disease severity (14–19). Importantly, $\text{oA}\beta$ levels in cerebrospinal fluid (CSF) are increased in AD patients versus controls; and are greater with *APOE4/4* versus *APOE3/3* in AD patients (20). Further pathology includes neuronal dysfunction, neuroinflammation, and cerebrovascular dysfunction (CerVD). 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* versus 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-*APOE* 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? The 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 A β deposition and clearance, neuroinflammation, tau pathology, neural plasticity, and CerVD (22–29). Further, many *APOE*/FAD-Tg models express h-apoE under heterologous promoters that may be activated by stressors [glial fibrillary acidic protein (GFAP) (27, 30)], or by cell types that are not the primary producers of apoE [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 h-*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 A β pathology as an example of AD phenotype. First, A β pathology is significantly delayed when FAD-Tg mice are crossed with *APOE*-KO mice, eliminating m-*APOE* (Table 1: compare A to B) (27, 30, 39, 40, 46). Second, A β pathology is further delayed when h-*APOE*-Tg mice are crossed with FAD-Tg/*APOE*-KO mice (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 delays the development of pathology to 12–16 months (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. The 5xFAD mice exhibit accelerated development of pathology, including amyloid deposition by 2 months (Table 1: compare A to D) (49–69). The 5xFAD mice were crossed with *APOE*-TR mice to produce the EFAD mice², which exhibit the anticipated delay in AD pathology, particularly with plaque development delayed to ~6 months (Table 1: compare D to E) (20, 66, 70–75), but still >6 months before the average FAD-Tg/*APOE*-Tg mice (compare Table 1: C and E).

Development of the EFAD mouse model

The 5xFAD mice coexpress five FAD mutations [amyloid precursor protein (APP) K670N/M671L + I716V + V717I and PS1 M146L + L286V] under the control of the neuron-specific mouse thymocyte differentiation antigen 1 theta (Thy-1) promoter (50). The specificity of this Thy-1 promoter for neuronal expression in the brain has been established (76, 77). The 5xFAD^{+/-} line, Tg6799, produces the highest amount of A β 42 (provided by R. Vassar, Northwestern University) (50). To establish colonies of EFAD mouse lines (E2FAD, E3FAD, and E4FAD), 5xFAD mice were bred to homozygous *APOE2*-, *APOE3*-, and *APOE4*-TR mice (32, 78) via a contract with Taconic Biosciences². Briefly, male (σ) *APOE*-TR^{+/+} mice on a C57/BL6 background were bred with female (f) 5xFAD^{+/-} mice on a C57BL6/B6 \times SJL background. The resulting female (f) mice, *APOE*-TR^{+/+}/5xFAD^{+/-} were backcrossed to male (σ) *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, 3**). Importantly, based on the design of the breeding strategy, the EFAD mice littermates (50%) are noncarriers (EFAD-NCs) for the 5xFAD transgenes and homozygous for *APOE* (5xFAD^{-/-}/*APOE*^{+/+}). Thus, EFAD-NCs provide both a comparison to the EFAD mice and a complementary approach to address functional questions about *APOE* in the absence of FAD-induced pathology.

Amyloid plaque deposition is not the same as soluble A β peptide

Amyloid plaques (together with NFTs) 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 A β vaccine trials (<https://endpts.com/mercks-leading-phiii-bace-drug-implodes-in-latest-alzheimers-disaster/>) and amyloid imaging studies (79–81). This has led to an apparent bias against

²The EFAD mice were made by contract to Taconic Biosciences via an Administrative Supplement (2005–2008) to M. J. LaDu, the Principal Investigator of Project 3 (Project Director: L. J. Van Eldik).

TABLE 1. Effects of h-*APOE* on A β pathology in FAD-Tg mice

Model	A β Pathology				
	<4 Months	4–8 Months	8–12 Months	12–16 Months	≥ 16 Months
(A) FAD-Tg		↑	↑↑	↑↑↑	↑↑↑↑
(B) FAD-Tg \times <i>APOE</i> -KO			↑	↑↑	↑↑↑
(C) FAD-Tg \times <i>APOE</i> -Tg				↑	↑↑
(D) 5xFAD ^{+/-}	↑↑	↑↑↑	↑↑↑↑	($\epsilon 4 > \epsilon 3$) ↑↑↑↑↑	($\epsilon 4 > \epsilon 3$) ↑↑↑↑↑↑
(E) 5xFAD ^{+/-} \times <i>APOE</i> -TR = EFAD-Tg		↑ ($\epsilon 4 > \epsilon 3$)	↑↑ ($\epsilon 4 > \epsilon 3$)	n.m.	n.m.

Differences in A β pathology (measured as A β levels using biochemical methods or A β deposition using immunohistochemistry) in FAD-Tg mice in the presence or absence of m- or h-*APOE* genotypes across the mouse lifespan among FAD-Tg (27, 33–35, 37–46, 327, 328) (A), FAD-Tg/*APOE*-KO (27, 30, 39, 40, 46) (B), FAD-Tg/*APOE*-Tg (27, 28, 30, 47, 48) (C), 5xFAD-Tg (50, 55, 58, 62–65, 68, 69, 222, 329–339) (D), 5xFAD/*APOE*-TR (EFAD-Tg mice) (20, 66, 70–75, 257) (E). A β pathology change: low \uparrow to high $\uparrow\uparrow\uparrow\uparrow\uparrow$, relative to an earlier age or among FAD-Tg mouse models. If measured and significant, differences between *APOE* genotypes are specifically indicated within each cell (as applicable). n.m., not measured.

amyloid plaques as a therapeutic target and even A β as a measure of AD pathology, evident from publications (82–85), press releases (<http://www.thetimes.co.uk/article/how-mice-immunity-is-hindering-research-n5fl2j5fj>, <https://www.theatlantic.com/health/archive/2017/02/alzheimers-amyloid-hypothesis/517185/>), documentaries (<http://www.monsterinthemind.com/>), and TEDx talks (<https://www.youtube.com/watch?v=6tBSFEzkk0A>). One aspect of this debate is the nature of the aggregation pathways for A β , particularly A β 42. As illustrated in **Fig. 1**, one paradigm for considering this assembly process is whether soluble oA β is either “on” or “off” pathway to the assembly of amyloid plaques (86). Via the on pathway, oA β 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 (Fig. 1A)? Alternatively, in the off pathway assembly process, A β can proceed via two separate processes, the pathogenic formation of oA β or formation of the traditional insoluble fibrils that continue to assemble into amyloid plaques, considered to be benign (Fig. 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 oA β , as the pathogenic target. This distinction is critical as FAD mutations indicate only that increases in A β 42 or the increase in the A β 42/A β 40 ratio cause FAD, not that the toxic assembly form of the peptide is amyloid plaques. Indeed, a novel FAD-Tg mouse model that promotes oA β formation, rather than fibrils and amyloid plaques, develops features of AD pathology, including tau hyperphosphorylation, neuroinflammation, synaptic alteration, cognitive deficits, and neuronal loss (87). For the purposes of this review, we consider that oA β 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

The 5xFAD mice versus EFAD mice

The EFAD mice are a tractable mouse model to study the development of a number of components of AD pathology

(Tables 2, 3). Although 5xFAD mice express m-apoE, which we argue is distinct from h-apoE in the proximal mechanistic pathways that lead to AD pathology, we have included a comparison of AD pathology in 5xFAD versus EFAD mice to demonstrate, in part, the rapid onset and accelerated development of AD symptoms in the 5xFAD mice (Tables 2, 3). In addition, pathology in 5xFAD mice can be used to predict pathology in the EFAD mice. A particularly useful example is neuronal loss, which is significant by 12 months in the 5xFAD mice, predicting that in the EFAD mice neuronal loss will likely occur at >16 months. 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 \geq E2FAD), as well as an emerging sex effect ($\text{♀} > \text{♂}$) on behavioral deficits, histopathology, and neuronal viability (Table 2), as well as A β 42 and apoE solubility, neurotrophic factors, and neuroinflammatory cytokines, but not with APP processing (Table 3). Thus, the EFAD mice allow for the monitoring of multiple AD-related symptoms through the mouse lifespan (Tables 2, 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, ♀ versus ♂ comparisons.

APOE modulated AD symptoms exhibited in EFAD mice

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) (**Figs. 2–5**). Cognitive decline in human AD patients (99) and the normal population follows $\epsilon 4 > \epsilon 3 > \epsilon 2$ (100), greatest in ♀ $\epsilon 4$ carriers (5, 101, 102). Similarly, in ♀ EFAD mice, cognitive impairment is E4FAD > E3FAD \geq E2FAD, as measured by Morris water maze (MWM), with the deficits increasing from 2 to 6 months (Fig. 2A) (70). In 8-month-old E3FAD and E4FAD mice, cognitive impairment is E4FAD > E3FAD and $\text{♀} > \text{♂}$, as measured by novel object recognition (NOR) and Y-maze (YM) (Fig. 2B) (71). Although cognitive testing

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

Model	Phenotype					
	<4 Months	4–8 Months	8–12 Months	12–16 Months	≥16 Months	
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, exploration and memory	—	↑ (MWM: ♀ε4 > ε3 ≥ ε2, 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.

5xFAD mice: *Behavioral deficits*: MWM (49, 50, 59–61, 340), YM (49, 51, 54, 55, 58), fear conditioning (FC) (52, 56, 57), NOR (329, 330), conditioned taste aversion (CTA) (52), cross-maze (CM) (62). *AD-related histopathology*: Aβ deposition: immunohistochemistry (IHC)-mAb to Aβ (50, 52, 55, 58, 62–65, 68, 329–337); plaque deposition: Thio-S (50, 64, 69, 332, 334); CAA: Thio-S, methoxy-X04 (334, 341); neuroinflammation: astrogliosis or microgliosis: IHC-mAb to GFAP or ionized calcium binding adaptor molecule 1 (Iba1)/F4-80 (50, 55, 58, 61, 65, 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, 329–331, 335, 336, 343–345); neuronal loss: cresyl violet, IHC-mAb to RNA binding protein, fox-1 homolog 3 (NeuN): *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-mAb to Aβ42-specific (MOAB-2) (66, 71) or IHC-mAb to Aβ (4G8) (73); plaque deposition: Thio-S (66, 71, 257); CAA: Thio-S (73); neuroinflammation: IHC-mAb to GFAP or Iba1 (72); pTau: Western blot for p-sites (75). *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.

in 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 with *APOE3* (20, 103, 104). For our initial characterization of the pathology in the EFAD mice, we used ♂ mice aged 2–6 months (Fig. 3) (66). Both Aβ accumulation and thioflavine-S (Thio-S)-positive plaque deposition begin in the subiculum (SB), followed by the deep layers of the frontal cortex (CX) (Fig. 3A, 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 to 6 months compared with the 5xFAD mice expressing m-*APOE*: 5xFAD > E4FAD > E3FAD ≥ E2FAD (Fig. 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 with 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 (Fig. 3C) (72). Further, there are higher levels of the pro-inflammatory cytokine, interleukin (IL)-1β, in E4FAD mice compared with E3FAD

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

Model		Biochemical Measures				
		<4 Months	4–8 Months	8–12 Months	12–16 Months	≥16 Months
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.	

5xFAD mice: *ApoE and Aβ solubility*: total Aβ: ELISA (49, 50, 52, 53, 57, 66, 222, 348), soluble Aβ42: in TBS or PBS extraction fraction by ELISA (67, 334, 338, 348), total apoE: ELISA or Western blot (67, 348, 349); TBSX-apoE: in TBSX extraction fraction by ELISA (67). *APP processing*: APP, BACE, C-terminal fragments by Western blot (49, 52, 53, 58, 67, 222, 329, 336, 337, 339, 348–352). *Neurotrophic factors*: brain-derived neurotrophic factor (BDNF) and pCREB proteins by Western blot or mRNA by qPCR (55, 61, 336, 350, 352, 353). *Neuroinflammatory cytokines*: TNF-α, IL-1β by qPCR (185, 329, 342, 351, 354). EFAD mice: *ApoE and Aβ solubility*: total Aβ: ELISA (66, 172), soluble Aβ42: in TBS extraction fraction by ELISA (66, 73, 172), total apoE: ELISA (66, 70), TBSX-apoE: in TBSX extraction fraction by ELISA (66, 172). *APP processing*: APP by Western blot (66). *Neurotrophic factors*: BDNF and pCREB proteins by Western blot (70, 193). *Neuroinflammatory cytokines*: TNF-α, IL-1β 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.

mice. 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 6 months, 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 with 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 tau (pTau) and NFTs, 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 7 months (Fig. 3D), not detectable at 3 months (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

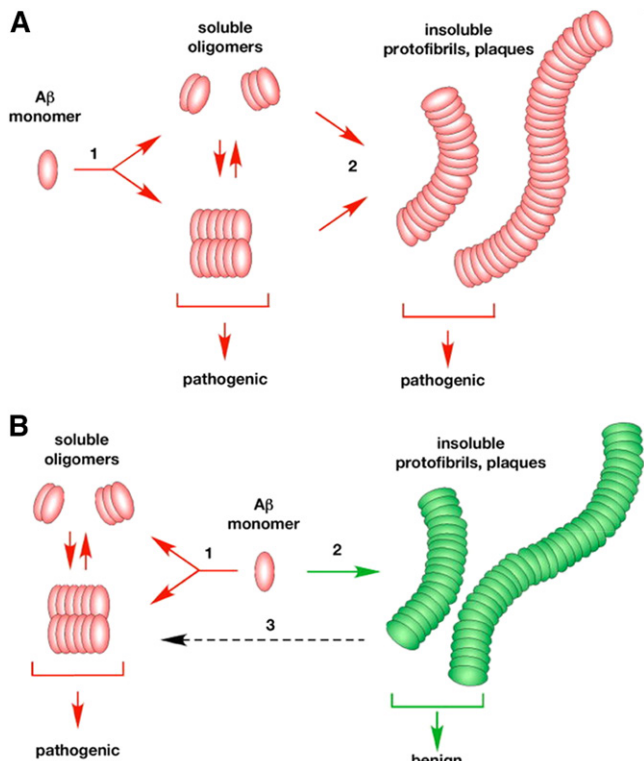


Fig. 1. A β assembly pathways: oA β on or off pathways for amyloid plaque deposition. A: Via the on pathway, oA β s can be pathogenic, but are structural precursors for the formation of the traditional insoluble fibrils that continue to assemble into the parallel β -sheet structure of amyloid plaques,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

that synaptic deficits may precede frank neuronal loss in humans (135–139), eventually resulting in the cognitive impairments characteristic of AD. Postsynaptic density protein 95 (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 to 6 months compared with E3FAD and E2FAD mice (Fig. 4) (70). These deficits in synaptic proteins are consistent with the behavioral deficits.

Solubility of apoE and A β 42 in ♂ EFAD mice. In brain homogenates, total A β 42 levels are 5xFAD \gg E4FAD > E3FAD = E2FAD (Fig. 5A) (66). To measure the solubility of both A β and apoE, particularly the apoE-lipoproteins in brain tissue, we adapted a three-step sequential protein extraction

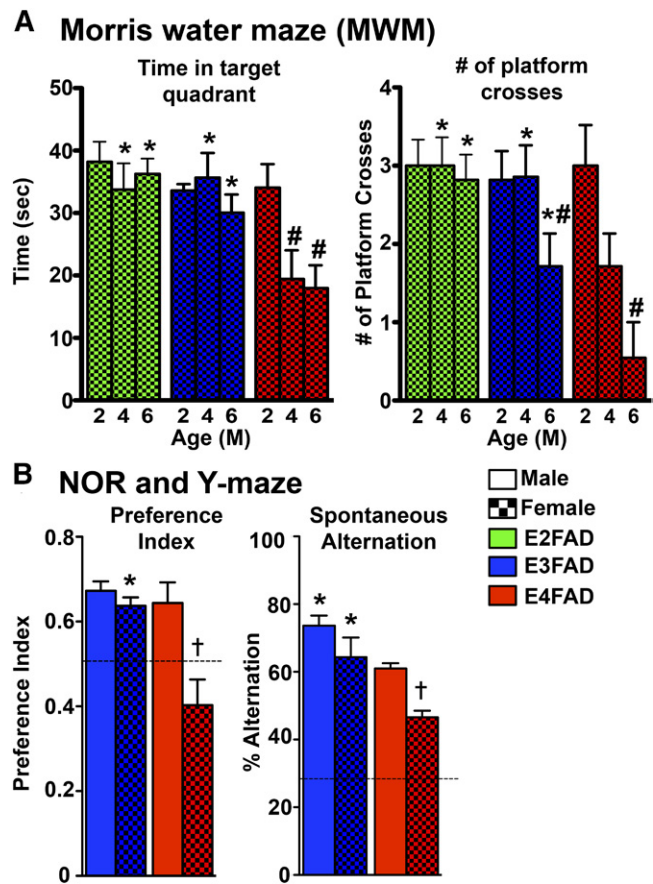


Fig. 2. Behavior deficits in EFAD mice. A: MWM in 2-, 4-, and 6-month-old ♀ EFAD mice, behavior performed during light cycle [adapted from (70)]. B: NOR preference index and YM spontaneous alternation in 8-month-old ♂ and ♀ EFAD mice, behavior performed during dark cycle [adapted from (71)]. $P < 0.05$: *versus E4FAD within sex, #versus 2 months, †♀ versus ♂ within genotype.

protocol using TBS, TBS + Triton X-100 (TBSX), and formic acid, producing extraction profiles for A β and apoE (Fig. 5B, D) (66, 67). In the A β extraction profile, the 5xFAD \gg E4FAD > E3FAD = E2FAD pattern was observed in the TBS and formic acid extraction fractions, while the TBSX fraction contained low levels of A β 42 that were comparable across the Tg-mouse strains [Fig. 5B, adapted from (66, 67)]. These biochemical results are consistent with the delay in A β deposition induced by the replacement of m-APOE with h-APOE (Fig. 3A), as are the genotype effects in levels of total and soluble A β 42 with E4FAD > E3FAD = E2FAD (Fig. 5A, B).

The apoE extraction profile detects apoE-lipoproteins in the TBSX fraction, a nonionic 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 (5, 47, 166–170), and EFAD mice (Fig. 5C), this isoform-specific difference is only in the TBSX-apoE4 (Fig. 5D) (66). C57BL/6 WT mice do not express A β 42, but do express m-apoE and, so, are included for comparison with the 5xFAD mice. For total apoE, the order is the

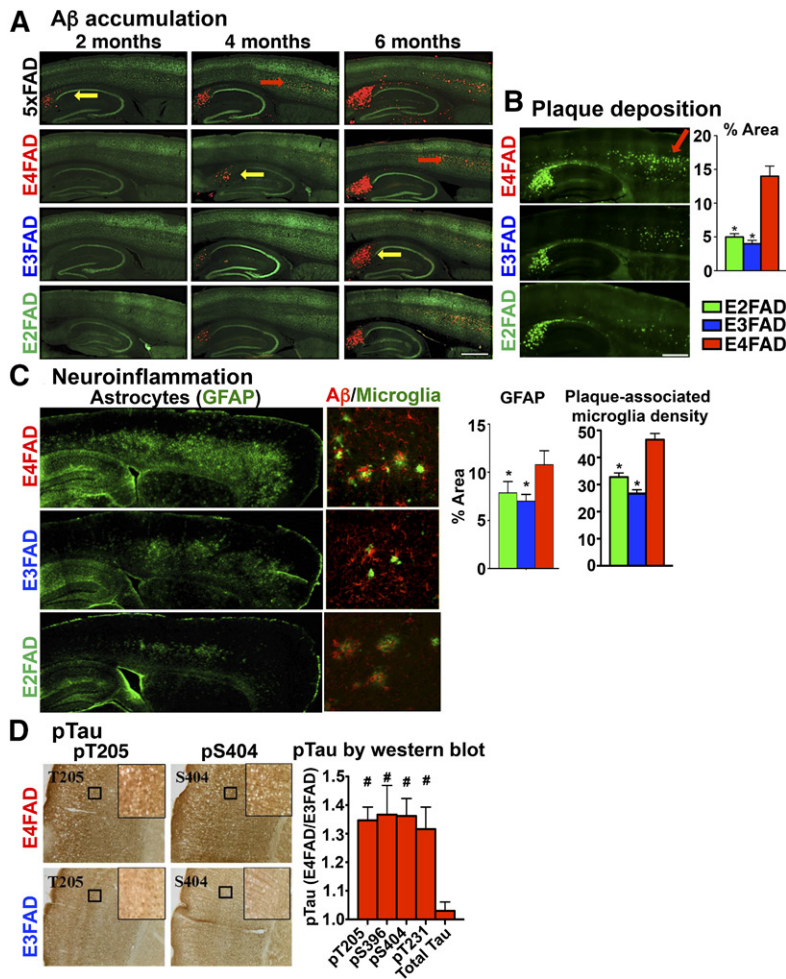


Fig. 3. AD histopathology in 2- to 6-month-old ♂ 5xFAD and EFAD mice. **A:** Total Aβ deposition by immunohistochemistry (IHC) in 5xFAD and ♂EFAD sagittal brain sections at 2, 4, and 6 months with mAb for Aβ (MOAB-2, red) and mAb for neurons [RNA binding protein, fox-1 homolog 3 (NeuN), green]. **B:** Plaque deposition: Thio-S staining in 6-month-old ♂ EFAD sagittal brain sections, quantified by percent area of CX [reproduced from (66)]. **P* < 0.05 versus E4FAD. **C:** Neuroinflammation: IHC staining in 6 month ♂ EFAD sagittal brain sections for astrocytes (GFAP) [adapted from (72)], quantified as percent area of CX; and for reactive microglia [ionized calcium binding adaptor molecule 1 (Iba1), green] and Aβ (MOAB-2, red), quantified as plaque-associated microglial density [reproduced from (72)]. **P* < 0.05 versus E4FAD. **D:** pTau: IHC in 7 month ♂ EFAD CX for phosphorylated Tau pT205 and pS404 sites. Insets: 20× magnification (black box). pTau quantified in EFAD CX by Western blot, expressed as E4FAD/E3FAD, #*P* < 0.05 versus E3FAD [reproduced from (75)]. Arrows for pathology: yellow = HP, red = CX.

reverse of the total Aβ levels: WT < 5xFAD ≅ E4FAD < E3FAD = E2FAD [Fig. 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 formic acid 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 (Fig. 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 see (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 monoclonal antibody (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* versus *APOE3* negatively correlate with soluble Aβ levels (Fig. 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 (PLs) in complex biological samples with relative ease. Recently, a lipidomics study revealed that the ratios of arachidonic acid (AA) and DHA increased in several major PL classes in the blood from cognitively normal ε4 carriers who converted to mild cognitive impairment/AD within 3 years (173). Longitudinal profiling of E3FAD and E4FAD mice showed that blood

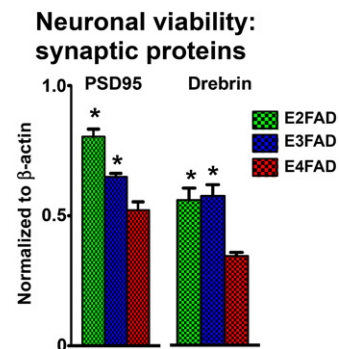


Fig. 4. Synaptic protein levels in ♀ EFAD mice. Synaptic proteins PSD95 and drebrin measured in 6-month-old ♀ EFAD mice by Western blot and normalized to β-actin [adapted from (70)], **P* < 0.05 versus E4FAD.

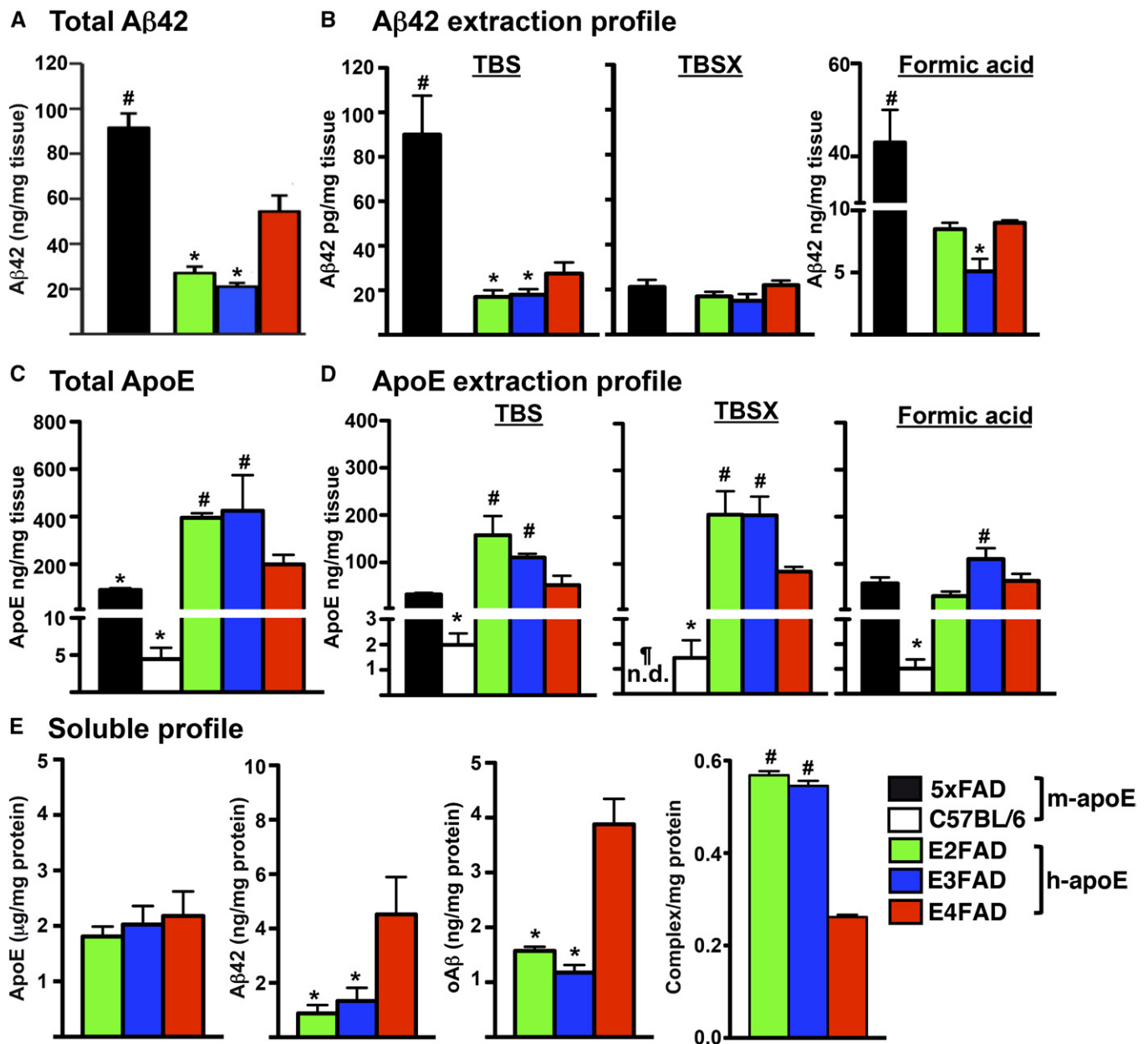


Fig. 5. Aβ42 and apoE solubility in 6-month-old ♂ 5xFAD, C57BL/6, and EFAD mice. A: Total Aβ42 levels in the brain. B: Aβ42 extraction profile after sequential extraction of CX with TBS, TBSX, and formic acid [adapted from (66, 67)]. C: Total apoE levels in the brain. D: ApoE extraction profile of CX, 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 ± SE normalized per milligram tissue (A–D) or per milligram protein (E).

AA- and DHA-containing PL species were altered as early as 2.5 months of age. At 6 months, AA- and DHA-containing lysophosphatidylcholine species increased in blood, but decreased in the brains of E4FAD compared with E3FAD mice (173). Previous studies have shown that lysophosphatidylcholine-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 to E3FAD and E2FAD mice. In addition, brain DHA transport is reduced in *APOE4*-TR mice compared

with *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

CerVD is reemerging as a key component of AD, with unresolved questions as to the extent of CerVD and its significance to cognitive decline. Additional issues include the apparent differences between CerVD 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 A β in CerVD.

Cerebrovascular leakiness and vessel coverage in ♂ and ♀ EFAD mice. Plasma protein extravasation into the brain is a key outcome of CerVD, particularly BBB capillary leakage. Here, we demonstrate that fibrinogen levels in the CX and SB in 8-month-old EFAD mice follow the order: ♀ E4FAD > ♀ E3FAD = ♂ E4FAD > ♂ E3FAD (Fig. 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 \geq ♀ E3FAD > ♀ E4FAD [Fig. 6B, C reproduced from (71)]. Thus, the combination of ♀ sex, *APOE4*, and A β induce pronounced BBB deficits that likely contribute to cognitive deficits. Indeed, high fibrinogen levels have been demonstrated in AD patients that are ϵ 4 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), follow the order: ♂ E3FAD > ♂ E4FAD \geq ♀ 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 and microbleeds in ♂ and ♀ EFAD mice. Cerebral amyloid angiopathy (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 (Fig. 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, A β is found attached to the outside of laminin, in the perivascular space between brain endothelial cells and laminin, and penetrating the vessel (Fig. 7B). These data raise the important general question of how A β in the brain interstitial fluid deposits as CAA. Identified interstitial 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 interstitial fluid, which is then cleared along paravascular spaces of large veins. We propose that our data are consistent with apoE4-induced impaired perivascular A β 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 with ♀. While one suggestion for this apparent difference between the EFAD mice and humans is that CerVD 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 ♀ versus ♂ ϵ 3/4 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 (Fig. 7C) (71). In AD, microbleeds are associated with ♂, higher blood pressure, lower CSF A β 42, 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 8 months, 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

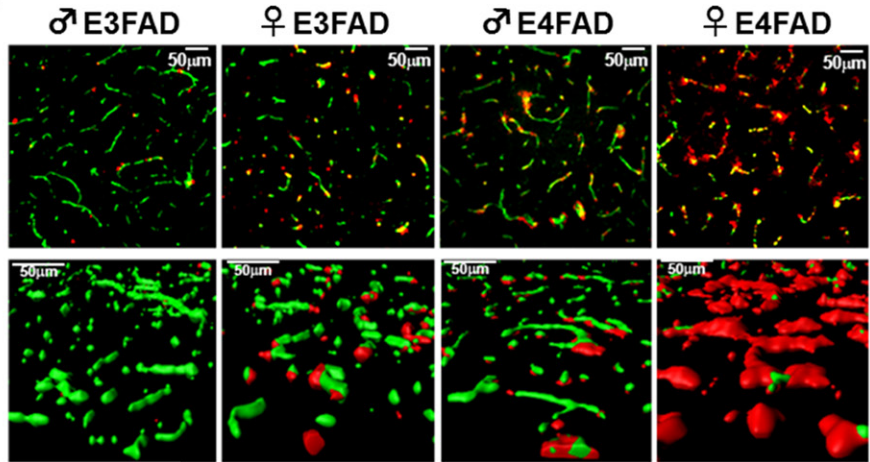
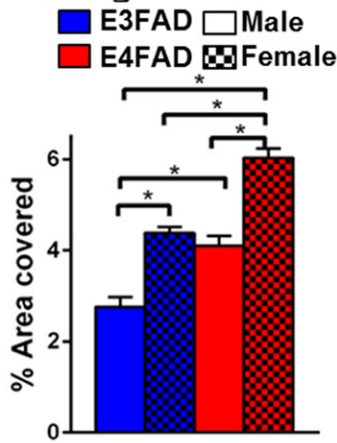
EFAD mice are a vital tool for testing therapeutics

EFAD mice exhibit temporally-defined, *APOE*-modulated changes in outcomes for efficacy (behavior, neuronal protein levels), pharmacodynamic activity (A β levels, neuroinflammation, and CerVD), 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 A β (e.g., sex hormone based). Further, whether a therapy is specific for *APOE4*, A β , or is applicable for all groups (*APOE* genotype, sex, \pm A β) can be determined by incorporating E2FAD, E3FAD mice, \pm FAD mutations (EFAD, EFAD-NC). Thus far, pharmacological and nonpharmacological therapies targeting apoE-lipidation, general neuroprotection, CerVD, and sex hormone pathways have been tested in EFAD mice.

Targeting apoE4 lipidation

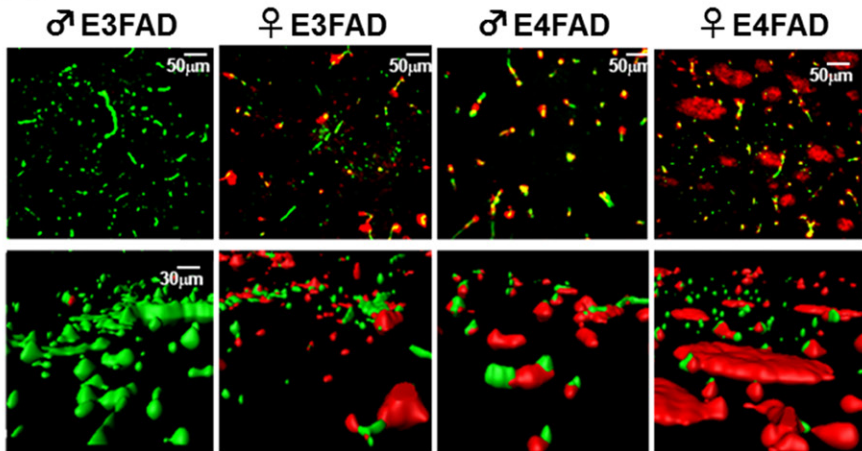
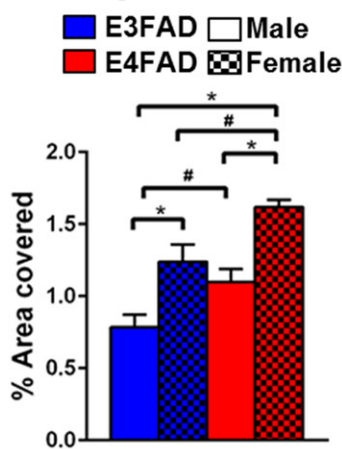
Lower lipidation/lipoprotein-associated levels in apoE4 was targeted in ♂ E4FAD mice using retinoid X receptor (RXR) agonists. The history of RXR agonists in the context of AD has been extensively reviewed elsewhere (184–192). Briefly, key issues center on whether RXR agonists increase apoE levels or lipidation (via increasing ABCA1 levels), the effect of human apoE4 and the duration of treatment. In ♂ E4FAD mice, short-term RXR agonist treatment (5.75–6 months) increased ABCA1 levels, apoE4 lipoprotein-association/lipidation, and apoE4/A β complex, decreased soluble A β , and increased PSD95 in the HP (172). However, RXR agonists induced no beneficial effects in ♂ E4FAD using a prevention protocol (5–6 months) and actually increased soluble A β levels in ♂ E3FAD and ♂ E4FAD CX with the short-term protocol, possibly the result of systemic hepatomegaly. These data support RXR agonists to address

A Fibrinogen levels in cortex



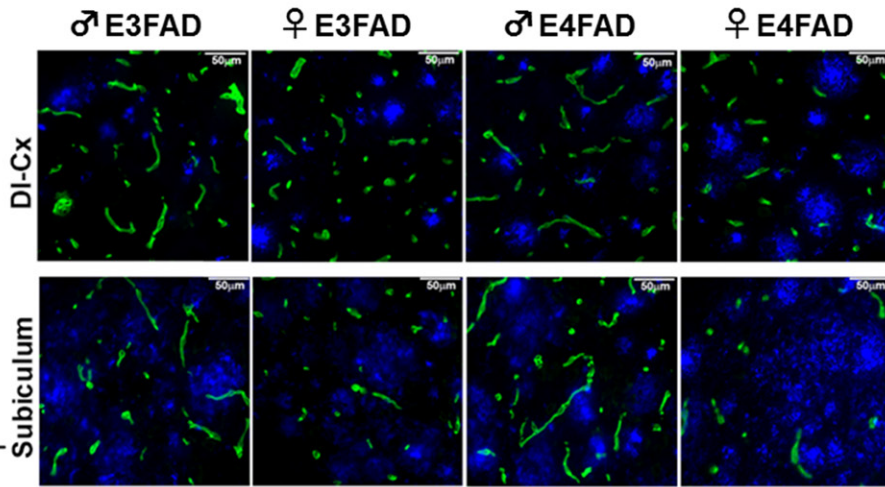
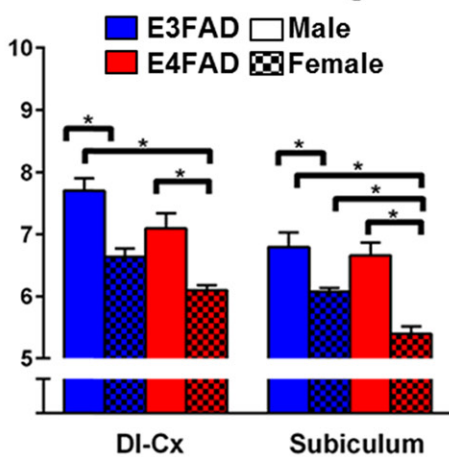
Red = Fibrinogen, green = CD31

B Fibrinogen levels in subiculum



Red = Fibrinogen, green = CD31

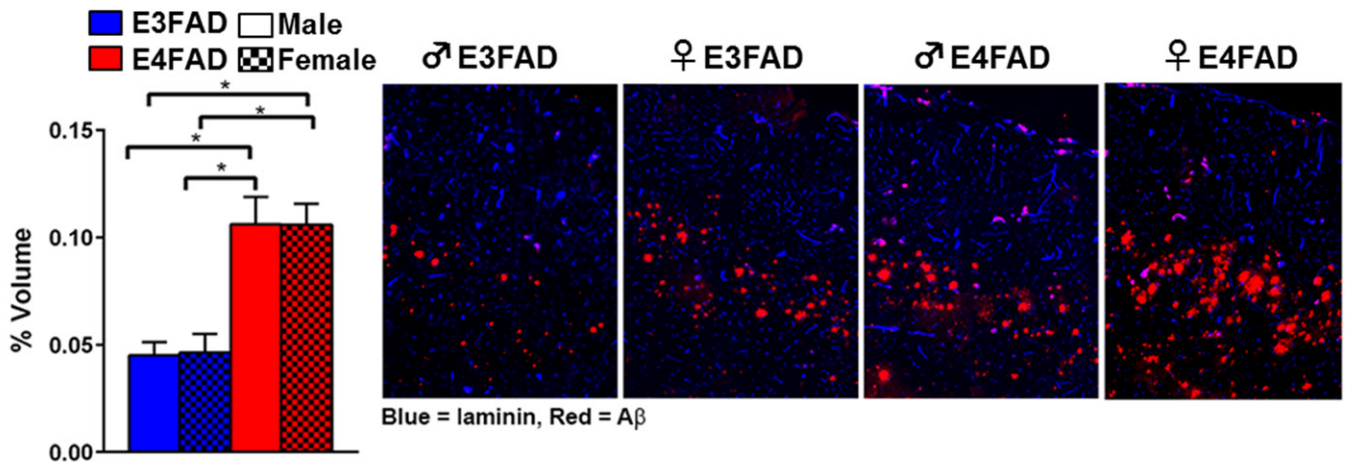
C Total vessel coverage



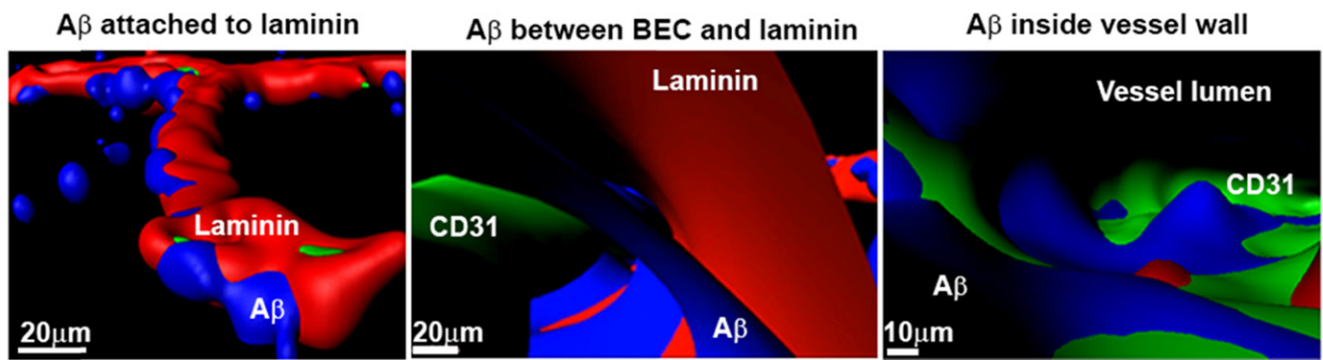
Green = laminin, blue = Aβ

Fig. 6. Cerebrovascular leakiness and vessel coverage in 8-month-old EFAD mice. Fibrinogen (red) levels in the CX (A) and SB (B) follow the order: ♀ E4FAD > ♀ E3FAD = ♂ E4FAD > ♂ E3FAD. Green = CD31; n = 8. Data expressed as mean ± SEM. **P* < 0.05 by two-way ANOVA and Tukey's post hoc comparisons. #*P* < 0.05 by two-way ANOVA 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)].

A CAA



B Examples of CAA-like deposition in ♀ E4FAD



Microbleeds

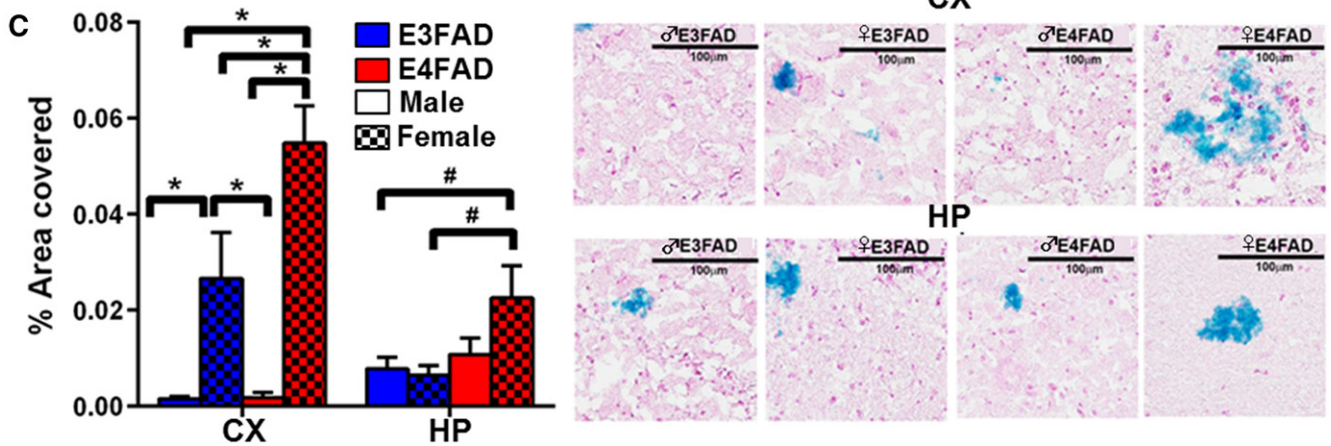


Fig. 7. CAA and microbleeds in 8-month-old EFAD mice. **A:** Cortical CAA-like deposition is highest in ♀ E4FAD and ♂ E4FAD; n = 8. Data expressed as mean ± SEM. **P* < 0.05 by two-way ANOVA and Tukey's post hoc comparisons (new data). **B:** Aβ deposits are found attached to laminin, in the perivascular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

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, 4-methyl-5-(2-(nitrooxy) ethyl) thiazol-3-ium chloride, was designed based on the anticonvulsant drug, Zenda, to activate the NO/cyclic guanosine monophosphate/phosphorylated cAMP-response element binding protein (pCREB) pathway as a multifunctional protective drug. 4-methyl-5-(2-(nitrooxy) ethyl) thiazol-3-ium

chloride treatment of ♂ E4FAD mice (3.5–6 months) lowered A β levels (soluble and insoluble) and increased both pCREB and PSD95 (193).

Nonpharmacological treatments

Additional nonpharmacological treatments tested in EFAD mice include EGF targeting cerebrovascular dysfunction (71) and 17- β estradiol (E₂) treatment of ovariectomized (OVX) ♀ EFAD mice. E₂ decreased soluble A β ₄₂ levels in ♀ E3FAD and ♀ E4FAD mice. However, insoluble A β levels increased in ♀ E4FAD mice (194). Therefore, the activity of E₂ may be dependent on the relative impact of extracellular and soluble A β on AD-induced neurodegeneration, with the results consistent with the hypothesis that soluble A β is toxic, while amyloid plaques are relatively benign (Fig. 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 A β 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 [(B6SJL/F1 \times C57BL/6) from 5x FAD (50) \times (C57BL/6) from *APOE*-TR (32)], we estimate that 10–14 months will represent middle age and 18 months will represent old age (<https://www.nia.nih.gov/research/dab/aged-rodent-colonies-handbook/strain-survival-information>) (196). Specifically, with the known survival rates for the background strains of the EFAD mice: 1) 5x FAD have a ~75% survival rate at 16 months (197); and 2) C57BL/6 and *APOE*-TR have 75% survival rate at 24 months for ♂ and 22 months for ♀ (196). Thus, the EFAD mice have ~75% survival at 20 months for ♂ and 19 months for ♀, making our target “old age” 18 months. Although specific measures of AD pathology in the EFAD are significant by 6 months, pathology continues to develop until at least 18 months, the oldest EFAD mice we have examined thus far (data not shown). 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, A β deposition, tau pathology, neuroinflammation, and neuronal viability (Table 2), as well as apoE lipidation and A β 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 versus 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 A β and amyloid deposition, as well as soluble A β levels are higher in ♀ versus ♂ in several FAD-Tg mice (Tg2576, APP/PS1, 3xTg-AD) (64, 198–203), as well as the EFAD mice (Table 3) (73). 2) In *APOE*-Tg mice, cognitive deficits are greater in ♀ *APOE4* versus *APOE3* [for review (21, 204–209)]. In EFAD mice, behavioral deficits are E4FAD > E3FAD and ♀ > ♂ in 6- and 8-month-old mice (Fig. 2, Table 2). 3) In humans, lifetime AD risk, cognitive decline and accumulation of A β is ♀ > ♂ in ϵ 4 carriers. These data suggest that the greatest risk for AD is with ♀ *APOE4* carriers (6–13, 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* versus sex will be determined by heterozygous E3/4FAD mice versus homozygous E3FAD and E4FAD mice, as established by age and AD pathology. For example, A β deposition, neuroinflammation, and tau pathology in ♂ E4FAD versus ♀ 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* versus 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 A β pathology at both 6 and 8 months, ♀ E3FAD \approx ♂ E4FAD at 8 months (71), suggesting a cliff or tipping point where ♀ sex is dominant compared with *APOE* genotype. However, unlike humans, as the ♀ EFAD mice age, they maintain 45–80% E₂ levels and normal uterine weight (214–218), which may produce an interesting phenotype at older ages with the scale tipping toward the dominance of *APOE* genotype. This change can be compared with OVX \pm E₂ 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 \leq 8 months. As ♂ and ♀ mice are aged from 10 to 14 to 18 months, sex and *APOE* genotype interact to induce significant differences in various components of AD pathology (data not shown). As well, all of our work thus far has been with *APOE*^{+/+}/*5xFAD*^{+/-} mice. As the *APOE* heterozygous genotypes are investigated (ϵ 2/3, ϵ 2/4, ϵ 3/4), 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 ϵ 2/2, ϵ 2/3, and ϵ 2/4, 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 ϵ 2 alleles [estimated: ϵ 2/2 at 0.4%, ϵ 2/3 at 8.8%, and ϵ 2/4 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 ϵ 2/2, ϵ 2/3, and ϵ 2/4 is perhaps a more subtle model to study the protective 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 ϵ 2/4 behave more like the risk ϵ 4 or the protective ϵ 2. 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 genome-wide association study, suggesting the presence of other genetic 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 (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 A β deposition, amyloid plaques and soluble oA β in ♀ E4FAD compared with ♀ E3FAD mice (257). This increased susceptibility of ♀ ϵ 4 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 A β , 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 the 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 an apoE mimetic peptide in the EFAD to evaluate its effect on apoE levels or apoE4 lipoprotein-association/lipidation and reduction of AD pathology.

Cerebrovascular dysfunction (CerVD). Many of the planned treatment strategies that target either the proximal or downstream processes modulated by *APOE* and sex will likely also target CerVD. Proximally, directly targeting the structural and functional deficits of apoE4 may ameliorate detrimental changes that cause CerVD (190, 292–294). Targeting downstream signaling pathways or the soluble mediators produced by *APOE*-modulated activated glia (astrocytes and microglia) and pericytes may ameliorate CerVD, 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 $\epsilon 4$ 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.

Selective estrogen mimics and selective estrogen receptor modulators. E_2 is key for ♀ vulnerability to *APOE4*-induced AD risk and pathology: OVX-induced loss of circulating E_2 in premenopausal 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 versus late menopause treatment (321). The controversial outcomes associated with timing could be addressed with the development of safe ET alternatives for the prevention and treatment of AD, potentially specific for the *APOE* genotype of patient. Based on the need for ET alternatives, we plan to study selective estrogen receptor modulators (322–325), or selective estrogen mimics (326) in \pm 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 A β 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 modulates 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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