



APOE modulates the effect of estrogen therapy on A β accumulation EFAD-Tg mice



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HIGHLIGHTS

- The interactive effects of APOE and ERT after OVX on A β accumulation were determined in EFAD mice.
- ERT decreased extracellular amyloid plaque and A β deposition with APOE2 and APOE3.
- ERT increased extracellular amyloid plaque and A β deposition, but lowered soluble A β 42 with APOE4.
- First *in vivo* evidence that hAPOE differentially regulates the effects of ERT on A β deposition.
- A β 42 levels may be a critical read-out for APOE4 carriers enrolled in ERT trials.

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ABSTRACT

The post-menopausal loss of estrogen is key in the increased incidence of Alzheimer's disease (AD) in women. However, estrogen therapy (ET) clinical trials have produced conflicting results. The APOE gene of apolipoprotein E (apoE) likely modulates the effects of ET in AD. APOE4 is the greatest genetic risk factor for AD, increasing risk up to 15-fold compared with APOE3, and the negative effect of APOE4 on AD risk and neuropathology is greater in women than men. The interactive effects of APOE and ET may converge on modulation of amyloid-beta (A β) levels, as independently both the loss of estrogen and APOE4 increases A β accumulation. Thus, in this study, 3-month old female EFAD mice (5XFAD mice crossed with apoE-targeted replacement mice), which express increased levels of A β 42 and human APOE were ovariectomized and treated for 3 months with either 17- β estradiol (OVX^{ET+}, 0.25 mg total) or vehicle control (OVX^{ET-}) and the effects on A β accumulation were determined. Compared to the OVX^{ET-} cohort, in the OVX^{ET+} cohort, extracellular amyloid and A β deposition in the hippocampus and cortex were decreased with APOE2 and APOE3, but were increased with APOE4 by IHC. Biochemical analysis demonstrated increased total and insoluble A β levels with APOE4, and decreased soluble A β 42 levels with both APOE3 and APOE4, after ET. These data suggest that ET administered at menopause may benefit APOE4 negative women by decreasing extracellular and soluble A β 42. However, for APOE4 carriers, the efficacy of ET will be dependent on the relative impact of extracellular and soluble A β on AD-induced neurodegeneration.

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1. Introduction

Alzheimer's disease (AD) is the most common form of dementia and over the age of 65, 2/3 of the cases afflict women [1]. The increased prevalence of AD in women remains even after adjusting for age and education levels [1]. Epidemiological analysis [18,24] and controlled clinical trials [11] indicate that the post-menopausal loss of estrogen is a major mechanism for the increased AD risk in women. However, data on the effect of hormone replacement therapy (HRT) or estrogen therapy (ET) on AD risk and cognitive decline are conflicting [5,13,19]. Thus far, identified-confounding factors include the timing and duration of ET and whether ET was used alone or in combination with progesterone.

An additional factor that may modulate the effects of ET in AD is the APOE gene. APOE4 is the greatest genetic risk factor for AD,

Abbreviations: A β , amyloid- β ; apoE, apolipoprotein E; AD, Alzheimer's disease; ET, estrogen therapy; FAD, familial AD; Thio-S, thioflavin S; HRT, hormone replacement therapy; OVX, ovariectomy; OVX^{ET-}, ovariectomy without ET; OVX^{ET+}, ovariectomy with ET.

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increasing the risk up to 15-fold, whereas *APOE2* decreases risk 4-fold, compared to *APOE3* [17]. Importantly, the negative effect of *APOE4* on AD progression may be more pronounced in women, as *APOE3/4* is reported to increase AD risk ~4-fold compared to *APOE3/3* in women, but not in men [2,12]. Further, in patients with mild cognitive impairment (MCI), *APOE4*-dependent cognitive impairment and hippocampal volume loss is greater in women than men [6]. Thus, *APOE4* exerts an adverse effect directly on AD risk and on markers associated with AD in post-menopausal women. Although these data indicate that ET treatment may be particularly efficacious at improving cognition in *APOE4* carriers however published data are inconsistent [7,8,14]. In a placebo-controlled trial/study [16], ET was associated with less cognitive decline in *APOE4* negative but not in *APOE4* positive women [20]. In partial contrast, the Nurses' Health Study demonstrated that ET provided no effects in older women, regardless of *APOE* genotype [9], and in follow-up analysis, HRT was associated with a worse rate of cognitive decline, an effect more pronounced with *APOE4* [8]. However, HRT has also been demonstrated to decrease the risk of dementia associated with *APOE4* [14], and to improve markers of accelerated aging in *APOE4* carriers [7]. Thus, preclinical studies are required to determine interactive effects of *APOE* and ET on AD relevant pathology.

One potential interactive effect of *APOE* and ET is modulation of amyloid beta-42 ($A\beta$) levels. $A\beta$ 42 is considered the proximal neurotoxin in AD. Two major forms include extracellular $A\beta$ 42 present in amyloid plaques, and soluble $A\beta$ 42, considered a major cause of cognitive impairment in AD. Independently, both *APOE4* and the loss of estrogen increase $A\beta$ levels. Compared to *APOE3*, *APOE4* increases the extracellular and soluble $A\beta$ levels in transgenic mouse models expressing familial-AD mutations (FAD-Tg) [22] and in AD patients [17]. Importantly, amyloid deposition is increased in middle age females compared to men, an effect increased by *APOE4* [4]. In APP23 mice, the loss of estrogen accelerates plaque formation [23] and in 3xTG mice, a FAD-Tg mouse that also expresses human Tau, ovariectomy (OVX) exacerbates extracellular $A\beta$ accumulation, an effect prevented with ET [3]. Although these data indicate that ET may lower $A\beta$ levels, an effect particularly beneficial with *APOE4*, there is no direct *in vivo* data. Therefore in this study EFAD mice, which express 5XFAD mutations and human *APOE* [22], were utilized to determine whether *APOE* modulates the effect of ET on $A\beta$ accumulation after OVX.

2. Materials and methods

2.1. EFAD mice and treatment protocols

All protocols follow the UIC Institutional Animal Care and Use Committee protocols (IUCAC number 11-121). EFAD mice are the result of crossing 5XFAD mice (APP K670N/M671L+I716V+V717I and PS1 M146L+L286V) and apoE-targeted replacement mice [22]. There are three lines of EFAD mice; E2FAD (*APOE2*), E3FAD (*APOE3*) and E4FAD (*APOE4*). All breeding and colony maintenance was conducted at Taconic labs as described in [22]. At Taconic laboratories, 3-month old EFAD mice received a bilateral ovariectomy (OVX) performed under anesthesia, and a pellet containing either 0.25 mg of a 90-day continual release of 17 β -estradiol (OVX^{ET+}, Innovative Research of America #NC-111) or placebo pellet (OVX^{ET-}) was implanted subcutaneously in the posterior aspect of the neck. At 5.75 months, animals were shipped to the University of Illinois, Chicago for tissue harvest at 6 months.

2.2. Tissue harvest

EFAD mice were anesthetized with sodium pentobarbital (50 mg/kg) and transcardially perfused with ice-cold PBS

containing protease inhibitors (Calbiochem, set 3). Brains were dissected at the midline, with the left hemi-fixed in 4% paraformaldehyde (PFA) for 48 h, followed by storage at 4 °C in PBS + 0.05% sodium azide (NaN₃) until use. Right hemi-brains were dissected on ice into cortex, hippocampus and cerebellum, snap frozen in liquid nitrogen, and stored at –80 °C until use [22].

2.3. Biochemical analysis of $A\beta$ 42 levels

Dissected brains were sequentially homogenized in PBS, followed by PBS containing 1% Triton X-100 (PBSX), and then 5 M guanidine (Gu) as described [21]. $A\beta$ 42 levels were measured (Wako) by ELISA and all data are expressed per mg of brain tissue.

2.4. IHC analysis

Brains were incubated using two sequential 30% sucrose solutions (in TBS) for 24 h each. For immunohistochemistry, tissues were sectioned on a freezing microtome at 40 μ m intervals and stored in cryoprotectant (30% glycerol, 30% ethylene glycol in PBS). Every 8th section for 6 consecutive sections was stained for either amyloid plaques (Thioflavin-S, Thio-S) or for $A\beta$.

2.5. Thio-S staining for plaques

Thio-S plaque staining and quantification was conducted by an investigator blinded to *APOE* genotype and treatment beginning with the lateral-most section in the region of interest (ROI) [22]. Sections were washed in TBS (6 \times 5 min), mounted on glass coverslips, allowed to dry, rehydrated in Milli-Q water for 2 min and stained in 0.1% Thio-S (dissolved in 50% EtOH + 50% 1 \times PBS) for 5 min in the dark. Tissue was destained in 80% EtOH (2 \times 5 min) in the dark and mounted with VectaShield fluorescence mounting media.

2.6. Immunohistochemistry for $A\beta$

Free-floating sections were processed for immunohistochemical staining as described in [22], using the anti- $A\beta$ antibody MOAB-2 (mouse IgG_{2b}, 1:1000 dilution of 0.5 mg/ml stock) an anti-NeuN antibody (mouse IgG₁, 1:1000 dilution, Chemicon), Alexa fluorophore-conjugated isotype specific secondary antibodies (diluted 1:200) and ProLong Gold antifade mounting media containing DAPI (Invitrogen).

2.7. Image analysis

Slides were mounted into a Nanozoomer whole slide scanner at the University of Chicago. The scanner was programmed to measure FITC, DAPI and Texas Red. Tissue sections were outlined with a marking tool and slides were scanned. Images were then imported into Image J and converted to 8-bit gray images. A threshold for signal was established and optimized. Image J was programmed to measure number, size and % area covered.

2.8. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis using GraphPad Prism version 4 for Macintosh. $p < 0.05$ was considered significant.

3. Results

3.1. Extracellular amyloid plaque and A β deposition is decreased with APOE2 and APOE3 but increased with APOE4, in ovariectomized EFAD mice treated with estradiol

To determine the interactions between APOE and ET on A β accumulation, female EFAD mice, which express increased levels of A β 42 and human APOE were utilized [22]. EFAD mice develop extracellular A β deposition that initiates in the subiculum and the deep layers of the frontal cortex. 3-month old female EFAD mice were ovariectomized (OVX), and treated for 3 months with an implant containing either 17- β estradiol pellet (OVX^{ET+}, 0.25 mg total) or vehicle control (OVX^{ET-}). This method models ET given at the beginning of the menopause, which represents a significantly indication of ET use in women. Comparing the ET dose and treatment duration between humans and the EFAD mice used for this study is complicated by a number of factors. For dose, the overall confounding factor is the pharmacokinetics of estradiol in mice vs humans, which is dependent on the drug formulation, route/method of administration, as well as absorbance and clearance parameters. However, 0.25 mg dose of 17-estradiol in mice corresponds to levels in pro-estrus phase [10]. This stage in mice is equivalent to the early follicular phase of the menstrual cycle in humans. Typically, most hormone therapies replace to follicular levels in humans using 100 mg patch of ET bi-weekly (for example [15]). That aging is not a linear process in either mice or humans makes it particularly problematic to compare duration equivalency of treatment, as it is necessarily based on age. Simplistically, female mice live 2.3 years and humans 86 years, a ratio of ~1:39. Thus, a 3-month treatment in mice is ~10 human years. Based on these calculations, 0.25 mg treatment over 3 months in EFAD mice is the equivalent to a 100 mg patch of ET bi-weekly for 10 years.

Although there is a debate as to the exact role of amyloid deposits in causing memory dysfunction in AD, extracellular amyloid deposits are one of the pathological hallmarks used for diagnosis of AD post-mortem, and amyloid brain imaging is increasingly utilized pre-mortem to aid in the diagnosis of AD. Amyloid plaques consist primarily of the A β 42 peptide, therefore amyloid plaque accumulation using Thio-S staining (Fig. 1A and C), and extracellular A β deposition, using the A β -specific antibody MOAB-2 (Fig. 1B and D) was assessed.

Compared to the OVX^{ET-} cohort, in the OVX^{ET+} cohort, extracellular Thio-S (Fig. 1A for representative image and Fig. 1C for quantification) and A β (Fig. 1B for representative image and 1D for quantification) accumulation was decreased with APOE2 and APOE3 (plaque count, size and % area covered) but increased with APOE4 (% area covered) in the cortex and hippocampus (data not shown). Indeed, the % area covered by Thio-S was decreased ~60% for E2FAD and E3FAD and increased by ~90% in E4FAD mice. Thus, after ovariectomy, ET with APOE4 increases extracellular A β and amyloid plaques in brain regions that are susceptible to relatively high (hippocampus) and intermediate (cortex) extracellular A β accumulation.

3.2. Total A β levels are increased with APOE4 in ovariectomized EFAD mice treated with estradiol

In order for a more in depth analysis of A β 42 levels in OVX^{ET-/+} EFAD mice, biochemical (BC) analysis was conducted. With all three APOE genotypes the levels of A β 42 were highest in the hippocampus, followed by the cortex, while the cerebellum has virtually no A β 42 (Fig. 2A) in the OVX^{ET-} group. Compared to OVX^{ET-}, in the OVX^{ET+} group, total A β 42 levels were increased in the hippocampus (Fig. 2B) and cortex (Fig. 2C) for APOE4, consistent with

the increased extracellular amyloid plaques and A β 42 deposition (Fig. 1), but were unchanged with APOE2 and APOE3.

3.3. Soluble A β 42 levels are decreased with APOE3 and APOE4 in ovariectomized EFAD mice treated with estradiol

Accumulating evidence over the last decade suggests that soluble A β in brain is the proximate cause in the initiation of AD development. Therefore, a 3-step biochemical extraction protocol was used to sequentially extract soluble (PBS), detergent (PBSX), and insoluble (Gu) A β 42. In the hippocampus (Fig. 2D), compared to OVX^{ET-}, in the OVX^{ET+} group soluble A β 42 levels were decreased and insoluble levels increased for APOE4, whereas A β 42 levels were unchanged with APOE2 and APOE3.

In the cortex (Fig. 2E), compared to OVX^{ET-}, in the OVX^{ET+} group, soluble A β 42 levels were decreased for both APOE3 and APOE4, whereas for APOE2, soluble A β 42 levels were low in both the OVX^{ET-} and OVX^{ET+} groups. Insoluble A β 42 levels were increased for APOE4 in the OVX^{ET+} group compared to OVX^{ET-}.

In both susceptible regions, the data are consistent with ET decreasing soluble A β 42 levels, but increasing insoluble A β with APOE4. In addition, with APOE3, ET decreased soluble A β in the cortex, a region susceptible to intermediate extracellular A β accumulation, but not the hippocampus.

4. Discussion

The increased risk of developing AD in women, and evidence that the post menopausal loss of estrogen may underlie these effects, has led to the hypothesis that ET is a potential treatment to prevent or treat AD. However, prospective analysis and clinical trials have produced conflicting results [5,13,19]. Upon further analysis, these studies appear consistent with the notion that ET should be initiated at the time of menopause to elicit beneficial effects. Future clinical trials (ELITE/KEEPS) may shed more light on this issue. An important, but understudied, factor that may modulate the therapeutic effects of ET is human APOE. However, it is unclear whether carriers of all human APOE genotypes will equally profit from ET, or whether ET should only be administered to certain APOE genotypes i.e. either APOE4 carriers or non-carriers [7–9,14,20]. This issue necessitates the need for preclinical ET studies in AD-relevant mouse models that express human APOE, using markers pertinent to human AD as read-outs. In this study, for the first time, the interactive effects of APOE and ET administered at the time of ovariectomy (OVX) on A β accumulation were determined in EFAD mice.

Previous data using FAD-Tg mice have demonstrated that the loss of estrogen increases, and ET treatment after OVX decreases, extracellular A β accumulation [3]. However, as these studies used FAD-Tg mice that express mouse APOE, which is structurally and functionally distinct from any of the human APOE genotypes, the relevance of these data are unclear. Thus, the first important finding from this study is that ET initiated at the same time as OVX decreased extracellular amyloid plaque and A β deposition with APOE2 and APOE3, and decreased soluble A β with APOE3 in the cortex, a region susceptible to intermediate A β accumulation. Although comparing preclinical with clinical data is not clear-cut, these data are seemingly in agreement with the Cardiovascular Health study, in which current estrogen use was found to diminish the risk of cognitive impairment compared with never users by 41% in APOE4 negative women [20]. One potentially cautionary point is that in the hippocampus, which is a brain region susceptible to high extracellular A β deposition in EFAD mice, ET did not lower soluble A β 42 levels with APOE3. Currently, there is debate in the AD field on the relative contribution of extracellular A β

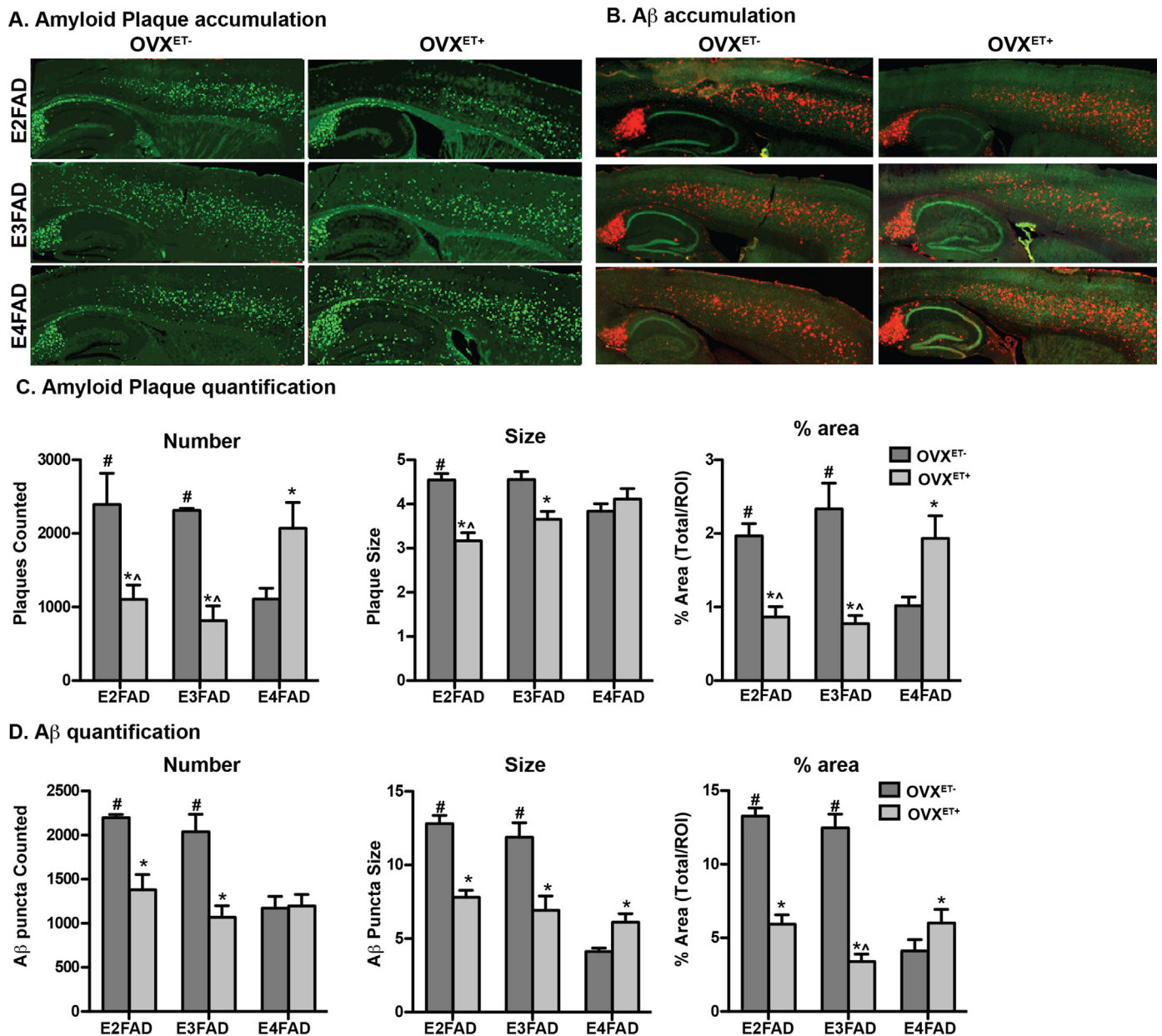


Fig. 1. Extracellular amyloid plaque and Aβ deposition is decreased with *APOE2* and *APOE3* but increased with *APOE4*, in ovariectomized EFAD mice treated with estradiol. Representative image of sagittal sections from ovariectomized EFAD mice treated with vehicle (OVX^{ET-}) or estradiol (OVX^{ET+}), and (A), stained with Thio-S or (B) immunostained for Aβ (red) and NeuN (green) (10× magnification). Quantification of number, size and % area covered in the frontal cortex of (C) Thio-S stained plaque plaques or (D) extracellular Aβ. Data are expressed as the mean ± S.E.M., and were analyzed by one-way ANOVA followed by Tukey's multiple comparison post hoc analysis. *n* = 5–6. **p* < 0.05 OVX^{ET-} vs OVX^{ET+}. #*p* < 0.05 vs E4FAD for OVX^{ET-}, ^*p* < 0.05 vs E4FAD for OVX^{ET+}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and soluble Aβ₄₂ to AD progression. If soluble Aβ is a significant contributor to neurodegeneration, our data may indicate that the efficacy of ET on cognition may require treatment in the preclinical stage of AD, where extracellular Aβ levels are lower. However, a 12-month placebo controlled study directly demonstrated a beneficial ET effect on cognition in *APOE4* non-carrier AD patients [16]. Tentatively, ET may prove beneficial for *APOE4* non-carriers for lowering extracellular Aβ and in prevention and treatment paradigms.

As *APOE4* [22] and the loss of estrogen, both increase amyloid deposition in FAD-Tg mice, a reasonable hypothesis is that ET should prove particularly effective at lowering Aβ in *APOE4* carriers. However, the second important finding of this study is that ET increased extracellular amyloid plaque and Aβ deposition, but lowered soluble Aβ₄₂ levels in E4FAD mice. As mentioned

previously, these data indicate that the efficacy of ET in human trials may depend on the relative contribution of soluble and extracellular Aβ to AD progression. However, the increased extracellular Aβ may be relevant for the findings that ET had no effect on cognitive impairment in *APOE4* carriers in the Cardiovascular Health study, and in a trial of ET in AD patients [16]. Furthermore, a recent prospective analysis has indicated that ET actually worsens rates of cognitive decline in *APOE4* carriers, although whether this effect is related to increased Aβ accumulation is unclear [8]. The results of this current study are particularly timely given the debate on the appropriate use of amyloid imaging. For *APOE4* positive post-menopausal women using ET, amyloid imaging and CSF Aβ₄₂ measurements may provide important information for interpreting the effects of extracellular plaques on cognition, and identifying

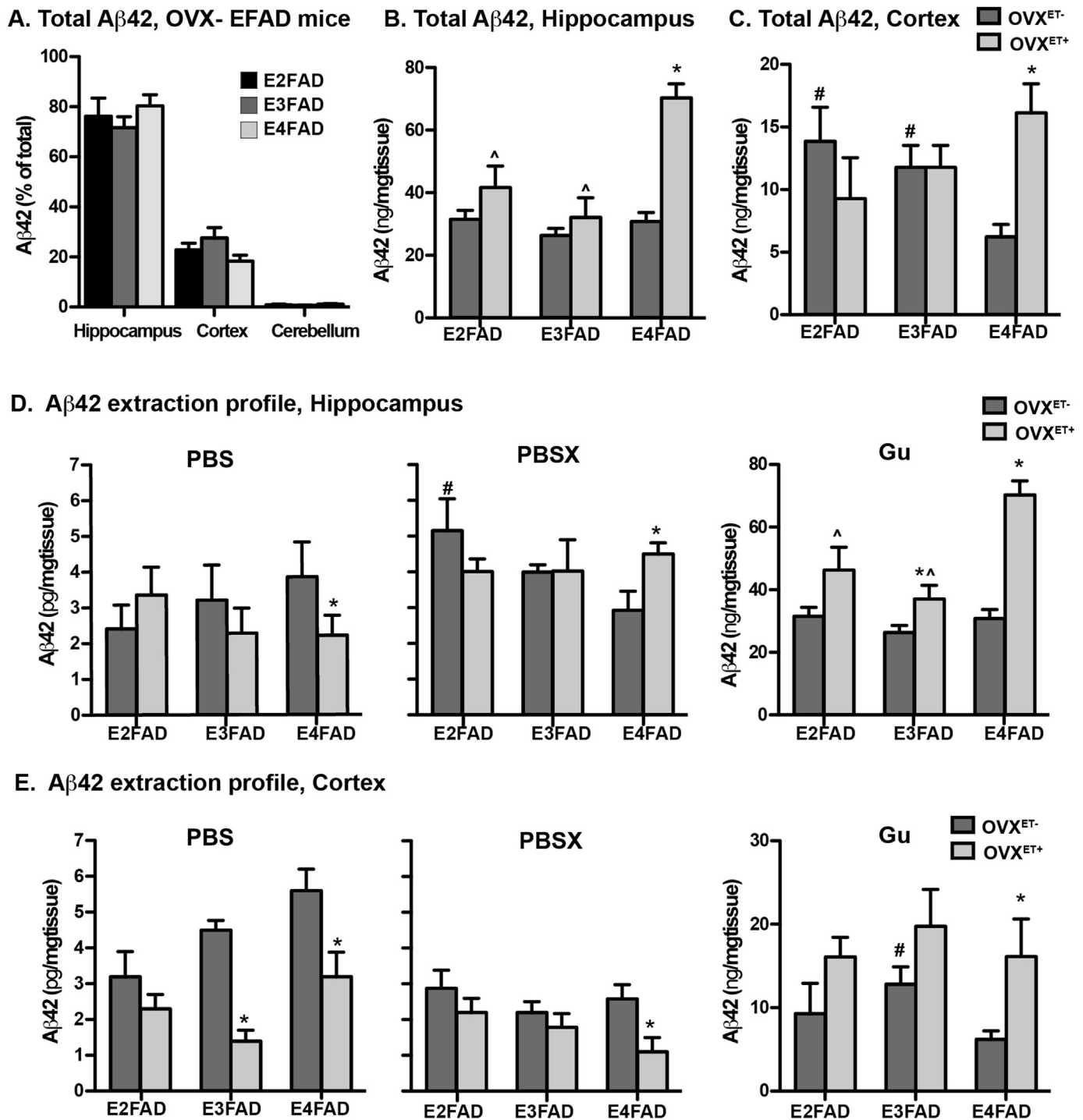


Fig. 2. Total and insoluble Aβ42 levels are increased with APOE4, whereas soluble Aβ42 levels are decreased with APOE3 and APOE4 in ovariectomized EFAD mice treated with estradiol. (A) Total Aβ42 levels in the CX, HC and CB of ovariectomized EFAD mice as measured by ELISA. Total Aβ42 levels in the (B) hippocampus and (C) cortex of ovariectomized EFAD mice treated with vehicle (OVX^{ET-}) or estradiol (OVX^{ET+}). Aβ42 extraction profile using a three-step sequential protein extraction (PBS, PBSX, Gu) in the (D) hippocampus and (E) cortex. Data are expressed as the mean ± S.E.M, and were analyzed by one-way ANOVA followed by Tukey's multiple comparison post hoc analysis. $n = 5-6$. * $p < 0.05$ OVX^{ET-} vs OVX^{ET+}. # $p < 0.05$ vs E4FAD for OVX^{ET-}. ^ $p < 0.05$ vs E4FAD for OVX^{ET+}.

potential high-risk groups for developing cognitive impairment and AD. Essentially, if an APOE4 carrier is enrolled in an ET clinical trial, then careful monitoring of amyloid levels could be incorporated in order to prevent future negative cognitive effects induced by Aβ.

This study focused on the effect of ET on Aβ accumulation for each APOE genotype. However, comparing APOE genotypes reveals

an interesting region-specific response to ET. It would be expected that in OVX^{ET-} EFAD mice, extracellular amyloid and Aβ would be highest with APOE4. However, in the cortex of OVX^{ET-} EFAD mice, extracellular amyloid, Aβ staining and Aβ42 concentrations were lower with APOE4 compared to APOE3 and APOE2, while levels in the hippocampus were equivalent among APOE genotypes. Furthermore, in the cortex, estradiol treatment raised extracellular plaque

and insoluble A β 42 levels in E4FAD mice to the levels in E3FAD OVX^{ET-}. Importantly, this was not observed in the hippocampus; insoluble A β 42 levels in OVX^{ET+} E4FAD mice were higher than OVX^{ET-} E3FAD and E2FAD mice. Thus, interactions between OVX and APOE on A β accumulation are likely dependent on the brain region, i.e. susceptibility to A β accumulation.

The interactive effects between ET and APOE are likely multifactorial, including effects, the cardiovascular system [20], cellular aging [7] and A β accumulation. Our ongoing studies are aimed at addressing these issues, including the potential mechanisms underlying these different effects, the therapeutic treatment window of ET and the effect of combined progesterone and estrogen HRT.

5. Conclusions

The present study provides the first *in vivo* evidence that human APOE differentially regulate the effects of ET on A β accumulation. Overall data indicate that ET may be beneficial with APOE2 and APOE3 for decreasing extracellular and soluble A β 42 however, the efficacy of ET treatment with APOE4 will be dependent of the relative contribution extracellular and soluble A β to neurodegeneration during AD progression.

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