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

Jacqueline Kunzler^{a,1}, Katherine L. Youmans^{a,2}, Chunjiang Yu^a, Mary Jo LaDu^a, and Leon Tai^a

^aDepartment of Anatomy & Cell Biology, University of Illinois at Chicago, Chicago, IL 60612

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+}, 0.25mg 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.

Keywords

Alzheimer's disease; Estrogen; Amyloid- β ; APOE; soluble A β ; AD transgenic mouse model

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

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Corresponding author: Mary Jo LaDu, University of Illinois at Chicago, Department of Anatomy and Cell Biology, 808 S.Wood St., M/C 512, Chicago, IL 60612, USA. Phone: 312-355-4795; Fax: 312-413-0354; mladu@uic.edu.

¹**Present address:**

Baxter Healthcare Corporation, Round Lake, IL 60073

²Department of Pharmacology, Boston University, Boston, MA 02215

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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, 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 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 *APOE2* [22], were utilized to determine whether *APOE* modulates the effect of ET on $A\beta$ accumulation after OVX.

MATERIALS AND METHODS

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 0.25mg 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.

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].

Biochemical analysis of A β 42 levels

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

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 β .

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.

Immunohistochemistry for A β —Free-floating sections were processed for immunohistochemical staining as described in [22], using the anti-A β antibody MOAB-2 (mouse IgG_{2b}, 1:1000 dilution of 0.5mg/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).

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.

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.

RESULTS

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+}, 0.25mg total) or vehicle control (OVX^{ET-}). This method models ET given at the beginning of the menopause, which represents a significant 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 biweekly (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 2.3y and humans 86y, a ratio of ~1:39. Thus, a 3-month treatment in mice is ~10 human years. Based on these calculations, 0.25mg treatment over 3 months in EFAD mice is the equivalent to a 100mg 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 (Figure 1A and C), and extracellular A β deposition, using the A β -specific antibody MOAB-2 (Figure 1B and D) was assessed.

Compared to the OVX^{ET-} cohort, in the OVX^{ET+} cohort, extracellular Thio-S (Figure 1A for representative image and 1C for quantification) and A β (Figure 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.

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 (Figure 2A) in the OVX⁻ group. Compared to OVX^{ET-}, in the OVX^{ET+} group, total A β 42 levels were increased in the hippocampus (Figure 2B) and cortex (Figure 2C) for *APOE4*, consistent with the increased extracellular amyloid plaques and A β 42 deposition (Figure 1), but were unchanged with *APOE2* and *APOE3*.

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 (Figure 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 (Figure 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 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.

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 and soluble A β 42 to AD progression. If soluble A β 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 A β 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 A β 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 A β in *APOE4* carriers. However, the second important finding of this study is that ET increased extracellular amyloid plaque and A β deposition, but lowered soluble A β 42 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 A β to AD progression. However, the increased extracellular A β 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 A β 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 A β 42 measurements may provide important information for interpreting the effects of extracellular plaques on cognition, and identifying 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 A β .

This study focused on the effect of ET on A β 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 A β would be highest with *APOE4*. However, in the cortex of OVX^{ET-} EFAD mice, extracellular amyloid, A β staining and A β 42 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.

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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Abbreviations

Aβ	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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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.

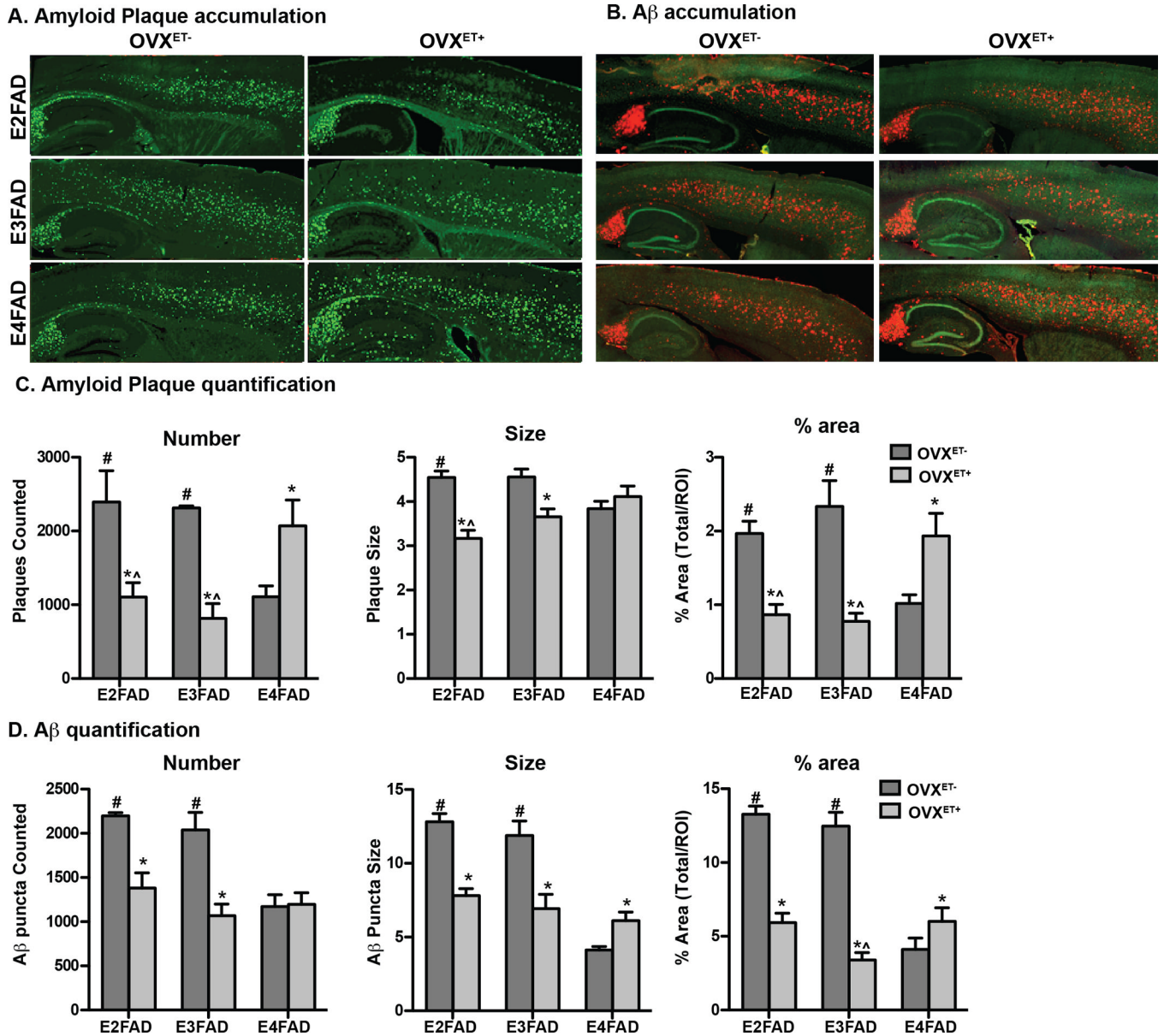


Figure 1. Extracellular amyloid plaque and A β deposition is decreased with *APOE2* and *APOE3* but increased with *APOE4*, in ovariectomized EFAD mice treated with estradiol
 Representative image of sagittal sections from ovariectomized EFAD mice treated with vehicle (OVX^{ET-}) or estradiol (OVX^{ET+}), and **A.** stained with Thio-S or **B.** immunostained for A β (red) and NeuN (green) (x10 magnification). Quantification of number, size and % area covered in the frontal cortex of **C.** Thio-S stained plaque plaques or **D.** extracellular A β . Data are expressed as the mean \pm 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-} versus OVX^{ET+}. # $p < 0.05$ vs E4FAD for OVX^{ET-}, ^ $p < 0.05$ vs E4FAD for OVX^{ET+}

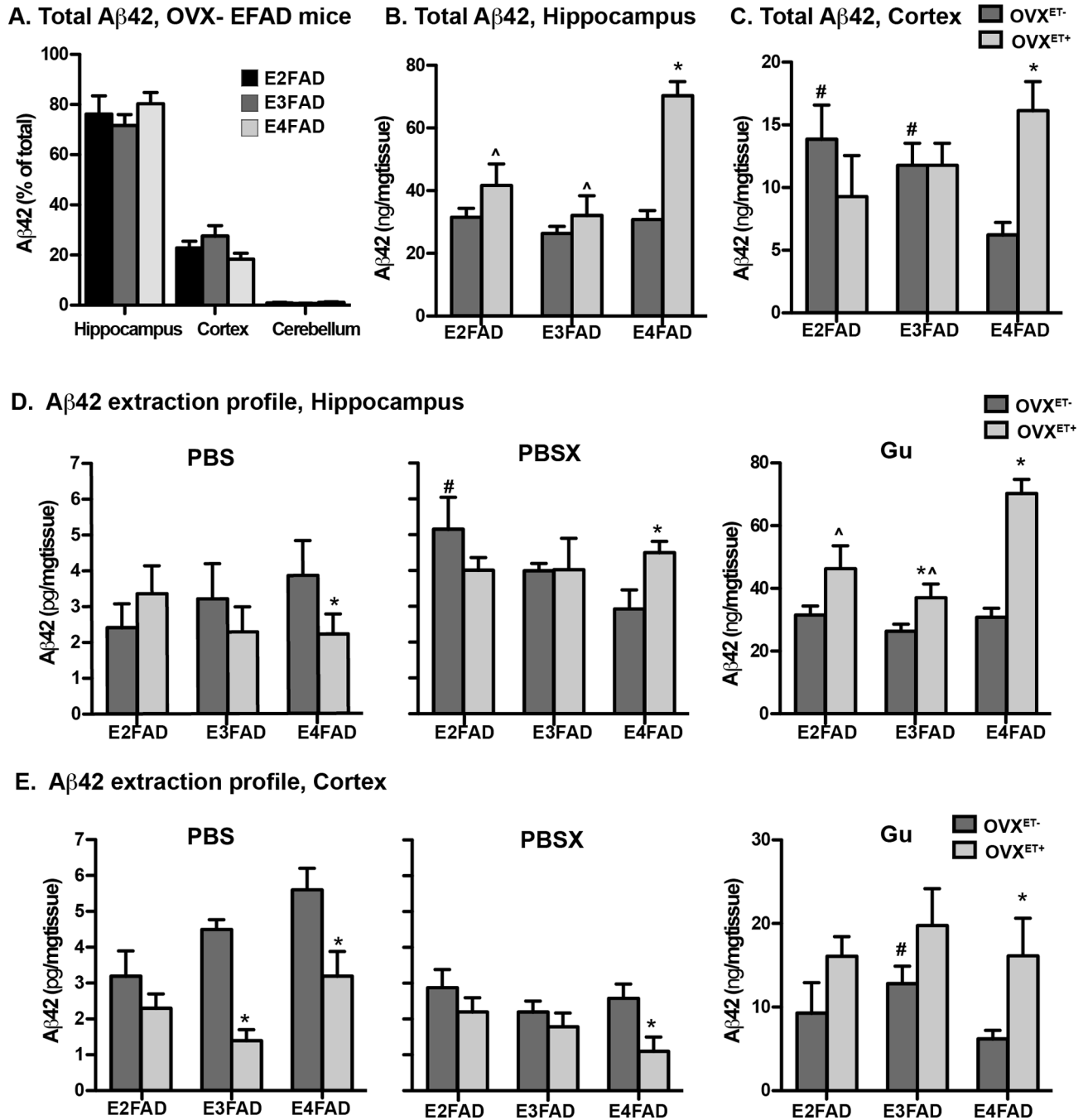


Figure 2. Total and insoluble A β 42 levels are increased with APOE4, whereas soluble A β 42 levels are decreased with APOE3 and APOE4 in ovariectomized EFAD mice treated with estradiol. **A.** Total A β 42 levels in the CX, HC and CB of ovariectomized EFAD mice as measured by ELISA. Total A β 42 levels in the **B.** hippocampus and **C.** cortex of ovariectomized EFAD mice treated with vehicle (OVX^{ET-}) or estradiol (OVX^{ET+}). A β 42 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 \pm 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-} versus OVX^{ET+}. # $p < 0.05$ vs E4FAD for OVX^{ET-}, ^ $p < 0.05$ vs E4FAD for OVX^{ET+}