Lack of hepatic apoE does not influence early Aβ deposition: observations from a new APOE knock-in model

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Abstract

Background: The apolipoprotein E (APOE) gene is the strongest genetic risk factor for late-onset Alzheimer disease (AD). ApoE is produced by both astrocytes and microglia in the brain, whereas hepatocytes produce the majority of apoE found in the periphery. Studies using APOE knock-in and transgenic mice have demonstrated a strong isoform-dependent effect of apoE on the accumulation of amyloid-β (Aβ) deposition in the brain in the form of both Aβ-containing amyloid plaques and cerebral amyloid angiopathy. However, the specific contributions of different apoE pools to AD pathogenesis remain unknown.

Methods: We have begun to address these questions by generating new lines of APOE knock-in (APOE-KI) mice (ε2/ε2, ε3/ε3, and ε4/ε4) where the exons in the coding region of APOE are flanked by loxP sites, allowing for cell type-specific manipulation of gene expression. We assessed these mice both alone and after crossing them with mice with amyloid deposition in the brain. Using biochemical and histological methods. We also investigated how removal of APOE expression from hepatocytes affected cerebral amyloid deposition.

Results: As in other APOE knock-in mice, apoE protein was present predominantly in astrocytes in the brain under basal conditions and was also detected in reactive microglia surrounding amyloid plaques. Primary cultured astrocytes and microglia from the APOE-KI mice secreted apoE in lipoprotein particles of distinct size distribution upon native gel analysis with microglial particles being substantially smaller than the HDL-like particles secreted by astrocytes. Crossing of APP/PS1 transgenic mice to the different APOE-KI mice recapitulated the previously described isoform-specific effect (ε4 > ε3) on amyloid plaque and Aβ accumulation. Deletion of APOE in hepatocytes did not alter brain apoE levels but did lead to a marked decrease in plasma apoE levels and changes in plasma lipid profile. Despite these changes in peripheral apoE and on plasma lipids, cerebral accumulation of amyloid plaques in APP/PS1 mice was not affected.

Conclusions: Altogether, these new knock-in strains offer a novel and dynamic tool to study the role of APOE in AD pathogenesis in a spatially and temporally controlled manner.

Keywords: apoE, Apolipoprotein E, Amyloid, Aβ, apoE particle, Cre-loxP, Albumin, Mouse model

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Background
Over the past 20 years, studies on apolipoprotein E (apoE) and its roles in various physiologic processes (atherosclerosis, Alzheimer disease – AD, etc.) have relied heavily on murine models that express the three main human isoforms (ε2, ε3, and ε4) under the control of the endogenous murine Apoe regulatory sequences [1–3]. These APOE knock-in mice were generated through targeted replacement strategies (referred to as APOE-TR mice from here onward) and have played instrumental roles in elucidating the isoform-specific differences in lipid metabolism and receptor binding affinity. In the context of AD, APOE modifies the risk for development of late-onset AD in an isoform-dependent manner (ε2 < ε3 < ε4, where the ε4 allele carries the highest risk) [4]. One mechanism through which APOE influences AD risk is through its effects on the metabolism of the amyloid-β peptide (Aβ), the main constituent of amyloid plaques found in AD patients. Indeed, crossing of transgenic mice that develop Aβ deposition in the brain (e.g. APP/PS1 or PDAPP mice that develop human-like Aβ plaques) to APOE-TR mice led to an isoform-dependent effect on cerebral amyloid plaque accumulation [5, 6], which is consistent with observations in humans [7]. Intriguingly, the effects of APOE on amyloidosis appear to be both isoform- and quantity-dependent, as reduction of apoE3 and apoE4 levels through genetic [8, 9] or pharmacologic [10] manipulations results in reduction of cerebral amyloid plaque load. While these studies shed important insights on one aspect of apoE’s role in AD pathogenesis, it remains unclear whether the effects resulted from a cell-independent or cell-autonomous mechanism.

Emerging data indicate that APOE not only affects AD risk, but also severity of pathology in dementia with Lewy bodies and neurodegeneration in tauopathies [11–14]. In particular, microglia-derived apoE appears to regulate the inflammatory response [11, 15–17], suggesting that the cellular source of apoE in both the brain and periphery has distinct functions in different diseases. In the brain, both astrocytes [18] and microglia [19] contribute to the pool of apoE. Additionally, apoE cannot cross the blood-brain barrier (BBB) [20], thus the pools of apoE in the central nervous system (CNS) and the periphery exist predominantly independently from one another. Some early studies in Apoe-deficient mice found age-dependent synaptic loss and learning deficits [21]. These deficits reflect the potential role of apoE in multiple physiologic processes responsible for maintaining brain homeostasis, including protection from oxidative damage [22, 23], maintenance of the BBB [24, 25], and cholesterol transport in the setting of synapse development [26] or neuronal injury [27]. Intriguingly, restoration of peripheral Apoe expression in an Apoe knock-out mice rescues the learning and memory deficit found in Apoe-deficient mice, despite exhibiting a similar degree of synaptic loss [28]. These findings suggest that both CNS and peripheral apoE (together with plasma lipids) are independent parameters that can affect neuronal function.

These and many other outstanding gaps in knowledge regarding apoE biology necessitate an experimental model where APOE expression can be specifically manipulated in different tissues and cell types. Here, we report the generation of an APOE knock-in mouse model where the various human APOE variants (ε2, ε3, and ε4) replace the endogenous murine Apoe locus (termed E2F, E3F, E4F mice individually, and APOE-KI mice collectively). Importantly, the human locus (specifically exons 2 to 4) is flanked by loxP sites that allow for the tissue-specific manipulation of APOE expression. We characterized the expression of apoE in the brain and brain cell types as well as the effects of APOE isoforms on Aβ deposition in this new model. We also investigated the effects of peripheral APOE knock-out (via hepatocyte-specific deletion of APOE expression using the Alb promoter) on plasma cholesterol homeostasis as well as Aβ deposition in the brain.

Methods
Contact for reagent and resource sharing
Further information and requests for resources and reagents should be directed to David M. Holtzman (holtzman@wustl.edu).

Experimental model and subject details
Targeting construct
The targeting strategy allows the generation of a constitutive humanization of the Apoe gene with the various human isoforms (APOE-ε2, APOE-ε3, and APOE-ε4), as well as a conditional knock-out and a constitutive knock-out of the gene. The targeting strategy is based on Ensembl transcripts ENSMUST00000174064 (mouse, corresponding to NCBI transcript NM_009696.3) and ENST00000252486 (human, corresponding to NCBI transcript NM_000041.3). The humanized alleles express the full length human proteins, including its signal peptide. Mouse genomic sequence from the translation initiation codon in exon 2 to the termination codon in exon 4 was replaced with its human counterparts: [Cys130, Cys176] for APOE-ε2, [Cys130, Arg176] for APOE-ε3, and [Arg130, Arg176] for APOE-ε4. Exons 2 to 4 (~3.9 kb) have been flanked by LoxP sites. A polyadenylation signal (hGHpA: human Growth Hormone polyadenylation signal) has been inserted to the 3’ of the genes (downstream of the distal loxP sites) in order to prevent transcriptional read-through. Positive selection markers were flanked by FRT (Neomycin resistance – NeoR) and F3 (Puromycin resistance – PuroR) sites and inserted...

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The text continues with experimental details, results, and conclusions related to the generation and characterization of the APOE humanized mouse model.
downstream of the proximal loxP site and upstream of the distal loxP site, respectively. The targeting vectors were generated using BAC clones from the mouse C57BL/6J RPCI-23 and human RPCI-11 BAC libraries.

**Generation of knock-in mice homozygous for human APOE isoforms (APOE-KI mice)**

Targeting vectors for the various human APOE isoforms were individually transfected into the Taconic Biosciences C57BL/6 N Tac ES cell line. Homologous recombinant clones were isolated using double positive (NeoR and PuroR) and negative (Thymidine kinase – Tk) selections. The constitutive humanized/conditional knock-out alleles were obtained after in vivo Flp-mediated removal of the selection markers. The newly introduced human APOE gene is expressed under control of the endogenous Apoe promoter. The resulting strains are referred to by their specific isoform expression (E2F, E3F, and E4F), or collectively as APOE-KI mice. The specific DNA sequence corresponding to each isoform (see Additional file 1: Figure S1) were verified through sequencing of exon 4 by GENEWIZ. DNA was isolated from fresh-frozen brain tissues, and exon 4 was amplified using specific primers (Forward: AACAACTGACCCCGGTGG; and reverse: GCTCGAACCAGCTCTTGAGG).

**Conditional knock-out of human APOE alleles**

To achieve tissue-specific knock-out of the human APOE alleles, APOE-KI mice were crossed to the appropriate strain with tissue-specific expression of Cre-recombinase. Specifically, peripheral knock-out of apoE was achieved by crossing Albumin-Cre (Alb-Cre) mice [29] (purchased from Jackson laboratory, strain # 003574, also known as B6.Cg-Speer6-ps1Tg(Alb-cre)21Mgn/J) to APOE-KI mice for two successive generations to obtain Alb-Cre mice homozygous for various APOE isoforms. All mice used in this study were maintained on a C57BL/6J background. Mice were subjected to experiments at either P7, P21, 1.5 months, or 3 months of age, per the various experimental designs. Mice were individually housed in AAALAC accredited facilities with temperature and humidity controls, and were under a 12-h light/dark cycle (lights on at 6:00 AM) cycle with free access to food and water ad libitum throughout all phases of the experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Washington University, and were in agreement with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC, WUSM).

**Method details**

**Brain extraction and preparation**

At the predetermined date of brain harvesting, the appropriate mice were anesthetized with intraperitoneal pentobarbital (200 mg kg – 1), and subsequently perfused with 3 U ml – 1 heparin in cold Dulbecco’s PBS for 3 min. The brains were then dissected carefully from the skull. The right hemisphere was fixed in 4% paraformaldehyde for at least 48 h before being transferred to 30% sucrose and stored at 4°C until they were sectioned. The left hemi-brain was dissected on an ice-cold stage into various parts (cortex, hippocampus, etc...), all of which were flash-frozen on dry ice and subsequently stored at −80°C until needed for biochemical analyses.

**Histology**

Following immersion in sucrose for at least 24 h, serial coronal sections (50 μm thickness) were collected from frontal cortex to caudal hippocampus (right hemisphere) using a freezing sliding microtome (ThermoFisher). Three hippocampal-containing sections (separated by 300 μm) from the right hemisphere of each brain were stained with biotinylated HJ3.4 (anti-Aβ1–13, mouse monoclonal antibody generated in-house, 1:500 dilution) [30] or biotinylated 3D6 antibody (anti-Aβ1–42) [31, 32] to visualize Aβ immunopositive plaques, as described previously. Microglia were immunostained using goat anti- IBA1 antibody (Abcam ab5076, 1:500 dilution). Astrocytes were immunostained using mouse anti-GFAP antibody (MAB3402, 1:1000 dilution). ApoE was immunostained with rabbit anti-apoE antibody (Cell signaling D719N, 1:500 dilution. All secondary antibodies were used in appropriate combinations depending on the primary antibody host, including: donkey anti-goat AF-488 (Invitrogen catalog # A-32814), donkey anti-rabbit AF-647 (Invitrogen catalog # A-31573), donkey anti-mouse AF-488 (Invitrogen catalog # A-21202), donkey anti-rabbit AF-568 (Invitrogen catalog # A-10042). All secondary antibodies were incubated at 1:500 dilution. Quantitative analysis of immunopositive staining was performed as described previously [33]. Briefly, images of immunostained sections were exported with NDP viewer (Hamamatsu Photonics). Using ImageJ software, images were converted to 8-bit grayscale, thresholded to highlight Aβ-specific staining and the percent area of a given brain region covered by thresholded staining calculated. For analyses of immunofluorescent staining (including GFAP, IBA1, apoE, X-34, and Aβ), 20X – 40X images were acquired on Nikon A1Rsi confocal microscope. Random z-stacks containing clusters of plaques were imaged, spanning approximately 30 μm of tissue in the z-plane with steps of 1.5 μm. Representative images are generated by projecting maximal intensity of each voxel on the same z-plane (using ImageJ software). All analyses were done blind to treatment and genotype.

**Real-time qPCR analysis**

RNA was extracted from frozen cortical tissue using TriZol (Life Technologies # 15596026) and purified using the
was used as a capture antibody. HJ5.1-biotin (anti-to as previously described [35, 36]. Cortices were dis- 

Mixed glial cultures were prepared from the cortex of E2F, E3F, and E4F neonatal mice (1–3 days old), similar to as previously described [35, 36]. Cortices were dis- 

RNeasy mini kit (Qiagen # 71404). Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Real-time qPCR was conducted with TaqMan primers (Life Technologies) and the TaqMan Universal PCR Master Mix (Thermo Fisher Scientific # 4304437) using the StepOnePlus ma- 

Brain homogenization

Brain cortices or hippocampi were sequentially homoge- 

Sandwich ELISA

The levels of Aβx-40 and apoE in PBS- and guanidine-soluble brain homogenates were measured by sandwich ELISA. For apoE ELISA, HJ15.3 and HJ15.7b were used as capture and detection antibodies, respect- 

Primary astrocyte and microglia cultures

Mixed glial cultures were prepared from the cortex of E2F, E3F, and E4F neonatal mice (1–3 days old), similar to as previously described [35, 36]. Cortices were dis- 

Non-denaturing gradient gel electrophoresis

Microglia-conditioned serum-free media and astrocyte-conditioned serum-free media samples were run on a 4– 

Western blot analysis

PBS-soluble brain lysates from the sequential homogenization step were analyzed for total protein con- 

Glutaraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level was used as a reference (Mm99999915_g1 Gapdh).

Seven milliliter of glial media was added to suspend the 

Western blot analysis

PBS-soluble brain lysates from the sequential homogenization step were analyzed for total protein con- 

probed with an anti-ApoE antibody (HJ15.7, 1:1000; in house). ApoE immunoreac- 

Biotin – 40 ELISA, HJ2 (anti-Aββ-35–40) was used as a capture antibody and for Aββ-37–42) was used as a capture antibody. HJ5.1-biotin (anti- 

Non-denaturing gradient gel electrophoresis

Microglia-conditioned serum-free media and astrocyte-conditioned serum-free media samples were run on a 4– 

Western blot analysis

PBS-soluble brain lysates from the sequential homogenization step were analyzed for total protein con- 

Tissue was digested in HBSS containing 0.25% trypsin – 40, Aββ-37–42 and apoE in PBS- and 

Western blot analysis

PBS-soluble brain lysates from the sequential homogenization step were analyzed for total protein con- 

Transcription Kit (Life Technologies). Real-time qPCR was performed using a High-Capacity cDNA Reverse 

Microglia-conditioned serum-free media and astrocyte-conditioned serum-free media samples were run on a 4– 

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PBS-soluble brain lysates from the sequential homogenization step were analyzed for total protein con- 

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dry-transferred onto a PVDF membrane using the iblot2 system (Life Technologies) and blocked with 5% milk in TBS-Tween (0.05%). The membrane was incubated with anti-apoE antibody HJ15.7 [34] (or HJ15.3) and anti-β-tubulin antibodies to probe for apoE and a loading control, respectively. Donkey-anti-mouse IgG-HRP was used as secondary antibody (Santa Cruz Biotechnology # sc-2096). All blots were developed for ~10 s using an enhanced chemiluminescence (ECL) Ultra kit (Lumigen TMA-6) and imaged on the SynGene Imager (BioRad) at the appropriate exposure.

Lipid measurements

Plasma Triglyceride (Wako Diagnostics catalog # 290–63,701), HDL (HDL-Cholesterol E, Wako Diagnostics catalog # 997–01301), and Total Cholesterol (Total Cholesterol-E, Wako Diagnostics catalog # 999–02601) concentrations were measured using kits from Wako Diagnostics adapted to half-area 96-well dishes (Corning, catalog # 3690). The protocols were performed according to the manufacturer’s specifications with the following adjustments. For the triglyceride and cholesterol measurements: half volumes of the samples and standards described in the provided microplate/microtiter methods were used. For the cholesterol measurements: a standard curve with levels of 0, 25, 100, 200, 397.4, 592.2 mg/dL was used. For the HDL measurements: 20 μL sample were mixed with an equal amount of Precipitating Reagent. Due to low sample HDL concentrations, samples were loaded at twice the volume of the standard curve. As a result, the derived sample concentrations were halved to reach the true value. After adding color reagent to the wells (150 μL/well), the dishes were gently mixed and incubated at 37 °C for ~5 min before reading. Absorbance at 600 nm and 700 nm were measured using a Cytation 5 Imaging Reader. Analysis was performed using Gen5 software and Microsoft Excel. Absorbance at 700 nm was subtracted from that at 600 nm to correct for contaminants. Samples and standards were measured in duplicate and triplicate, respectively. Sample concentrations were derived from a linear regression fit to the standard curve. For analysis, the blank absorbance was counted as the 0 mg/dL level (it was not subtracted). Samples were subjected to ~1–3 freeze-thaw cycles.

Quantification and statistical analysis

Statistics

All values are reported as mean ± SEM. A one-way ANOVA was used to test significance between more than two groups (Figs. 5, 6, 7, 8, and 9), and Tukey’s post-hoc test was used to test for differences between each of the groups. A two-way ANOVA was used to assess significance between more than two groups in the presence of additional variables (Figs. 5, 6, 7, 8, and 9), and Tukey’s post-hoc test was used to test for differences between each of the groups (Figs. 5, 6, 7, 8, and 9), with the exception of Fig. 7c – h, where Holm-Sidak multiple comparisons testing was used due to non-normal distribution of data in some groups. All statistical analyses were performed using Prism software (GraphPad). p < 0.05 is considered significant for all tests. No statistical analysis was used to determine sample size a priori. The sample sizes chosen are based on those used in previous studies from our laboratory. The number of samples indicates biological replicates as indicated in each of the figure legends. At most one outlier was removed per genotype via the Grubb’s test (alpha = 0.05, GraphPad QuickCalcs).
humanized allele expresses the full-length human APOE protein, including its signal peptide, rather than a fusion protein between the mouse signal peptide and the human mature protein. To verify accuracy and successful creation of the model, brain samples from all 3 lines were submitted for sequencing of exon 4 of the APOE locus by GENEWIZ, which confirmed the presence of human sequence and appropriate single nucleotide polymorphisms (SNPs) specific
for each isoform. Further details on the specific design of the vector can be found in the methods section.

We first characterized the newly created APOE-KI strains by confirming the presence of human APOE mRNA expression in the mice with qPCR analysis (Additional file 1: Figure S1e and S1f). At 3 months of age, there was a slight, but statistically significant, elevation in APOE mRNA level in the hippocampus of E4F mice compared to E2F or E3F mice (Additional file 1: Figure S1e). We also assessed APOE mRNA levels in whole brain hemispheres. Again, APOE mRNA levels were similar between genotypes, although APOE mRNA levels were slightly lower in E2F mice compared to either E3F or E4F mice, both of which were statistically significant (Additional file 1: Figure S1f). Next, we biochemically assessed the apoE protein levels in the brains of APOE-KI mice at 21 days and 3 months after birth. At 21 days of age, apoE protein levels in the cortex were significantly higher in E2F mice relative to those found in E3F or E4F mice (Fig. 1f). At 3 months of age, there was no difference in PBS-soluble apoE protein concentration between E2F and E3F mice, while E4F mice had significantly lower levels relative to E2F mice (Fig. 1g). We also measured guanidine-soluble apoE and found E3F mice to have significantly higher levels than E2F mice (Fig. 1h). Lower apoE protein levels in 3-month-old E4F mice were also seen via Western blot analysis of the same brain lysates utilizing HJ15.7 as the detecting antibody (Fig. 1i). This latter finding was replicated using another anti-apoE antibody (Additional file 1: Figure S1g). These data demonstrated that the newly generated APOE-KI mice expressing different apoE isoforms have similar levels of human APOE mRNA in the brain. Some differences in protein levels (higher apoE2, lower apoE4) are likely secondary to differences in protein stability, half-life, and metabolism, as has been seen in previous APOE knock-in mice [6, 37–44].

Human APOE is expressed in astrocytes and microglia in APOE-KI mice

The majority of apoE molecules in the CNS are synthesized by astrocytes [18], with a small portion coming from microglia [19]. We further characterized the expression pattern of apoE in the brain of APOE-KI mice by co-staining for apoE and traditional markers for astrocytes as well as microglia. We confirmed the presence of apoE protein in astrocytes by co-staining for apoE and the astrocytic marker GFAP (Fig. 2a). There was some apoE staining in cells with the morphology of astrocytes that were GFAP-negative. We also assessed microglia for the presence of apoE protein by co-staining for the microglial marker IBA1, however, we did not observe significant overlap of apoE and IBA1 signal (Fig. 2b). For simplicity, only representative images from E4F mice are shown, as similar findings were found in E2F and E3F mice.

ApoE’s role in AD pathogenesis was first recognized when apoE was found to co-localize with amyloid plaques, specifically at the center (i.e. the “core”) of mature, fibrillar amyloid plaques [45, 46]. ApoE expression is low in microglia under basal, homeostatic conditions, but is strongly up-regulated in the setting of various neurodegenerative insults [17, 47–49]. Thus, we investigated whether apoE can be found in microglia in the setting of amyloidosis, specifically in the APP/PS1–21 model which develops Aβ deposition in amyloid plaques beginning at 6–8 weeks of age [50]. APP/PS1–21 mice were crossed with APOE-KI mice for two successive generations and the brain sections from 4-month-old APP/PS1–21 mice homozygous for human APOE alleles (ε2/ε2, ε3/ε3, or ε4/ε4) were < 12 nm in size, E4F astrocytes did appear to produce a small, but notable, amount of approximately 8 nm-sized particles (Fig. 4). Microglia-conditioned media contained apoE particles that were overall much smaller than the astrocyte-derived particles. For E3F and E4F microglia, the majority of particles produced were about 8 nm in size with a small amount of particles 10–17 nm in size (Fig. 4).
However, for E2F microglia there did appear to be a shift in the relative amount of 10–15 nm-sized particles versus 8-nm-sized particles. While E2F microglia did produce a considerable amount of ~8 nm-sized particles, more 10–15 nm-sized particles were present than what was seen for E3F and E4F microglia. As larger particles contain greater amounts of cholesterol and phospholipid, these findings suggest that microglia secrete poorly lipidated apoE relative to the larger HDL-like lipoproteins secreted by astrocytes. These results highlight the need for future studies to more closely examine the properties of these apoE-containing particles and whether they also differ in their normal function as well as in pathological states.

**APOE isoform-dependent effect on Aβ accumulation in APP/PS1/EKI mice**

While APOE may influence AD pathogenesis in several ways, one of the major mechanisms is via its effect on Aβ accumulation in the brain, specifically on Aβ seeding and clearance. As previous APOE knock-in mice have been shown to influence Aβ deposition in an isoform-dependent fashion [5, 6, 8, 9, 43, 56, 57], we wanted to assess the effects of the major human APOE isoforms in the new APOE-KI model. Specifically, we investigated the effect of different human APOE alleles on Aβ accumulation in APP/PS1–21 transgenic mice. In this model, overexpression of the amyloid precursor protein (APP) in neurons leads to cerebral accumulation of Aβ-containing plaques that resemble those found in AD brains [50]. We crossed APP/PS1–21 mice on a C57BL/6 background with either E2F, E3F, or E4F mice on a C57BL/6 background to obtain APP/PS1–21 transgenic mice on an **APOE-ε2, ε3, or ε4** background (APP/PS1/E2F, APP/PS1/E3F, and APP/PS1/E4F mice, respectively) that do not express murine Apoe (collectively referred to as APP/PS1/EKI mice). We first measured the amount

![Fig. 2 Human APOE is expressed in astrocytes in APOE-KI mice.](image)
of PBS-soluble apoE in the cortex of APP/PS1/EKI mice via ELISA (Fig. 6a) and found overall apoE protein levels to be similar to those seen in APOE-KI mice. ApoE protein concentration in the cortex of female APP/PS1/E4F mice was slightly, albeit statistically significantly, higher than those found in female APP/PS1/E3F mice.

Since the APP/PS1–21 mice have visible neocortical plaque deposits beginning around 2 months of age [50], we assessed plaque accumulation in APP/PS1/EKI mice at 4 months of age, when sufficient plaques are present in the neocortex to allow for quantitative assessments. We first examined cerebral plaque load histologically by staining brain sections with an anti-Aβ antibody HJ3.4b (Fig. 5a). Quantitative analysis of the area covered by HJ3.4b staining in the cortex showed independent, but significant, effects of sex and APOE isoform. Post hoc analysis comparing apoE isoform within each sex did not find a statistically significant difference, although there was a trend towards elevated X-34 staining in apoE4-expressing female mice compared to apoE2 and apoE3. The relative differences in cortical burden of Aβ and X-34 pathology between different isoforms are very similar to what we observed when APP/PS1–21 mice were crossed to APOE-TR mice (Additional file 2: Figure S2a and S2c) and in our published data [8]. Interestingly, there was no effect of sex on Aβ pathology in this latter model (Additional file 2: Figure S2b and S2d).

To characterize the factors that account for the differences in Aβ pathology, we assessed the plaque density and average plaque size in APP/PS1/EKI mice. For Aβ staining, there was a significant effect of sex and APOE genotype, but no interaction. Post hoc analysis showed an increase in plaque density in female APP/PS1/E4F compared to APP/PS1/E3F mice (Fig. 5c). There was a significant effect of sex, but not of APOE genotype, on average plaque size (Fig. 5d). For X-34 staining, there was a significant effect of sex and APOE genotype (but no interaction) on plaque density. Post hoc analysis comparing APOE genotype within each sex found a
significant increase in plaque density in female APP/PS1/E4F mice compared to APP/PS1/E3F or APP/PS1/E2F mice (Fig. 5e). There was a significant effect of sex, but not APOE genotype on average X-34 plaque size. No differences were detected on post hoc analysis between any subgroup (Fig. 5f).

To further assess the total amount of Aβ accumulation, we measured the amount of PBS-soluble and PBS-insoluble (guanidine fraction) Aβ40 and Aβ42 in the cortex of APP/PS1/EKI mice. We observed a significant increase in PBS-soluble Aβ40 and Aβ42 in APP/PS1/E2F mice compared to APP/PS1/E3F mice (Fig. 6b and c) and significantly higher levels of Aβ42 in APP/PS1/E4F compared to APP/PS1/E3F mice (Fig. 6c). In the guanidine fraction (PBS-insoluble fraction) where the majority of Aβ accumulates, we detected a significant increase (~2-fold) in insoluble Aβ42 in APP/PS1/E4F mice relative to APP/PS1/E3F mice (Fig. 6d and e). There were no statistically significant differences between the other groups.

Our findings are consistent with previous studies where APOE-TR mice were crossed to various models of amyloidosis [58] and recapitulate (at least in part) the allele-dependent effect of APOE on amyloid deposition found in humans.

**Plasma lipid alterations in mice lacking liver-derived apoE**

Previous studies from our lab and others showed that complete ablation [59–63] or reduction [8–10] of apoE levels results in a decrease of Aβ pathology, particularly a marked reduction of fibrillar Aβ in the brain. However, it was difficult to assess the contribution of peripheral apoE ablation to phenotypes found in the brain in complete

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**Fig. 4** Qualitative assessment of microglia and astrocyte-derived apoE particles. Conditioned media samples from E2F, E3F, and E4F-derived primary cultures enriched for microglia and astrocyte were subjected to non-denaturing 4–20% Tris-glycine gradient gel electrophoresis followed by Western blotting. Approximate hydrated radius of marker proteins, run on the same gel, are shown for comparative purposes. Data shown are representative of 3 independent cultures from different cohorts of mice.
knock-out models. Thus, it remains unclear whether peripherally-derived apoE (or lack thereof) exerts any effect on cerebral amyloid pathology when apoE is still present in the brain. To this end, we took advantage of the newly created APOE-KI lines and investigated whether a lack of the main source of peripheral apoE in the blood, (i.e. the liver), can influence Aβ pathology in the brain.

While apoE is synthesized by a number of different organs outside of the CNS, the liver accounts for most circulating apoE [64]. Thus, we set out to ablate APOE expression in the liver through Cre-loxP technology, targeted through the hepatocyte-specific Alb promoter (that normally controls albumin expression). Specifically, mice expressing Cre-recombinase under the Alb promoter (Alb-Cre mice) [29] were crossed with APOE-KI mice for two successive generations to obtain Alb-Cre mice on all three human APOE backgrounds (AlbCre-EKI). The AlbCre-EKI mice were subsequently crossed with APP/PS1/EKI mice.
PS1/EKI mice to achieve hepatocyte-specific knock-out of APOE in APP/PS1/EKI mice (APP/PS1/E2F Cre, APP/PS1/E3F Cre, APP/PS1/E4F Cre mice, and APP/PS1/EKI Cre mice collectively). Littermates lacking Cre-recombinase expression (Cre−/−) serve as controls, which are effectively APP/PS1/EKI mice. We first confirmed successful deletion of APOE from hepatocytes by performing qPCR analysis from liver tissue, which showed undetectable levels of APOE mRNA in Cre-expressing mice (Fig. 7a). We next measured apoE protein from liver tissue and also found the levels to be markedly decreased in Cre-expressing mice, regardless of APOE genotype (Fig. 7b). APP/PS1/E2F mice also had significantly more apoE protein in the liver relative to APP/PS1/E3F or APP/PS1/E4F mice, despite similar hepatic expression of APOE mRNA.

We also collected the plasma from APP/PS1/EKI Cre mice and investigated the effect of Alb-Cre on the levels of apoE protein as well as the major lipid species. Due to the observed effect of sex on Aβ pathology in APP/PS1/EKI mice (Figs. 5 and 6), the males and females from this cohort were analyzed independently. Assessment of apoE levels in the plasma of male APP/PS1/EKI Cre mice through an ELISA assay showed a significant effect of APOE genotype and Cre expression, with a significant interaction (Fig. 7c). Plasma apoE levels in female mice were also influenced by APOE genotype and Cre expression, with a significant interaction between the latter two parameters (Fig. 7d). We performed post hoc pair-wise comparisons between male Cre+/− and Cre−/− groups, and found Cre expression significantly decreased plasma apoE levels across apoE isoforms regardless of sex (Fig. 7c and d). Notably, residual amounts of apoE protein were detected in both male (Fig. 7c) and female (Fig. 7d) APP/PS1/E2F Cre mice.
Fig. 7 (See legend on next page.)
likely because apoE2 has a low affinity for the LDL receptor.

As apoE plays an essential physiologic role in peripheral lipid homeostasis, we next examined the levels of total cholesterol (TC), triglycerides, and HDL in the plasma of APP/PS1/EK1Cre mice. For these analyses, male and female were analyzed separately, and plasma samples from mice with global deletion of murine Apoe (EKO mice) were included for comparative purposes (n = 5 males, 3 females). Our analyses of male mice showed a significant effect of both APP/PS1 and EKO Cre on plasma TC, with a significant interaction between these two parameters. Post hoc pair-wise comparisons between Cre+/- and Cre−/− groups found Cre expression to significantly increase plasma TC levels in male mice expressing apoE2, but not apoE3 or apoE4-expressing mice (Fig. 7e). Similarly, significant effects of APP/PS1 and Cre expression on plasma TC were found in female mice, with significant interaction between APP/PS1 and Cre genotypes. Post hoc pair-wise comparisons between Cre+/- and Cre−/− groups found Cre expression to significantly increase plasma TC levels in apoE2-expressing mice, but not apoE3 or apoE4-expressing mice (Fig. 7f). In both male and female mice, the TC level in APP/PS1/E2FCre mice is of greatest magnitude, and appeared to be comparable to those found in EKO mice (though the EKO mice were not included in the direct statistical analysis).

Plasma triglyceride concentrations in male mice follow a similar trend with a significant effect of APP/PS1 genotype and Cre expression, with a significant interaction. Post hoc pairwise comparisons between Cre+/- and Cre−/− groups found Cre expression significantly increased triglyceride levels in mice expressing apoE2 (p = 0.0001) or apoE3 (p = 0.0001), but not apoE4 (p = 0.1971). (n; E2; Cre+/-: 5, Cre−/−: 5; E3; Cre+/-: 5, Cre−/−: 5; E4; Cre+/-: 5, Cre−/−: 5, Cre−/−: 5, Cre−/−: 5; E4; Cre+/-: 5, Cre−/−: 5, Cre−/−: 5, Cre−/−: 5, Cre−/−: 5). Total plasma triglyceride concentrations in male mice were quantified. There was a significant effect of apoE isoform (F2,90 = 31.14, p < 0.0001) and Cre expression (F1,45 = 86.12, p < 0.0001) and a significant interaction (F2,45 = 24.10, p < 0.0001). Post hoc pairwise comparisons between Cre+/- and Cre−/− groups found Cre expression significantly increased plasma cholesterol levels in mice expressing apoE2 (p = 0.0001) or apoE3 (p = 0.0001), but not apoE4 (p = 0.1971). (n; E2; Cre+/-: 5, Cre−/−: 5; E3; Cre+/-: 5, Cre−/−: 5; E4; Cre+/-: 5, Cre−/−: 5, Cre−/−: 5, Cre−/−: 5, Cre−/−: 5; E4; Cre+/-: 5, Cre−/−: 5, Cre−/−: 5, Cre−/−: 5, Cre−/−: 5). Total plasma cholesterol in male mice was quantified. There was a significant effect of apoE isoform (F2,90 = 46.39, p < 0.0001) and Cre genotype (F1,45 = 24.31, p < 0.0001) with a significant interaction (F2,45 = 12.59, p < 0.0001). Post hoc pairwise comparisons between Cre+/- and Cre−/− groups found Cre expression significantly increased plasma cholesterol levels in apoE2-expressing mice (p < 0.0001), but not apoE3 or apoE4-expressing mice (n; E2; Cre+/-: 5, Cre−/−: 5; E3; Cre+/-: 5, Cre−/−: 5; E4; Cre+/-: 5, Cre−/−: 5, Cre−/−: 5, Cre−/−: 5, Cre−/−: 5, Cre−/−: 5, Cre−/−: 5). Total plasma HDL in male mice was quantified. There was a significant effect of apoE isoform (F2,49 = 43.03, p < 0.0001) and Cre expression (F1,41 = 61.81, p < 0.0001) with a significant interaction (F2,41 = 9.54, p = 0.0003). Post hoc pair-wise comparisons between Cre+/- and Cre−/− groups found Cre expression significantly decreased plasma apoE levels across apoE isoforms. (apoE2, p < 0.0001; apoE3, p = 0.0112; apoE4, p = 0.0223). (n; E2; Cre+/-: 5, Cre−/−: 5; Cre+EKO mice are also plotted according to sex (n; E2; Cre+/-: 5 males, 3 females), but were not included in statistical comparisons.)

The changes in plasma lipid composition found in APP/PS1/EK1Cre mice have some similarities and some differences compared to what was found in EKO mice. Though no direct statistical comparison was performed,
the incongruence in some of the dataset relative to the EKO control could reflect the effect of residual extrahepatic APOE expression (e.g. in macrophages) that is below the detection limit of our ELISA assay.

Liver-derived apoE does not influence Aβ accumulation in the brain

We next examined the effects of hepatocyte-specific APOE deletion on cerebral Aβ accumulation. Again, due to the observed effect of sex on Aβ pathology in APP/PS1/EKI mice (Figs. 5 and 6), the males and females from this cohort were analyzed independently. Brain sections from 4-month-old APP/PS1/EKICre mice and littermate controls were assessed for Aβ immunostaining with HJ3.4b antibody (Fig. 8a, b, and c). Quantitative analyses in both male (Fig. 8g) and female (Fig. 8h) mice found a significant effect of APOE genotype, but not Cre expression on the cortical area covered by Aβ staining. To further characterize the nature of the deposited Aβ plaques, brain sections were stained with X-34 dye which only stains fibrillar plaques (Fig. 8d, e, and f). Quantitative analyses of the area covered by X-34 staining showed similar trends to those found in HJ3.4b staining, with a significant effect of APOE genotype, but not Cre expression, in both male (Fig. 8i) and female (Fig. 8j) mice.
Fig. 9 (See legend on next page.)
To examine whether Alb-Cre expression alters apoE protein levels in the brain of APP/PS1/EKICre mice, we performed ELISA assays on cortical brain homogenates. In the PBS-soluble fraction from male mice, there was a trend towards a significant effect of APOE genotype, but no significant effect of Cre genotype, on apoE protein levels (Fig. 9a). In female mice, there was no significant effect of APOE or Cre genotype (Fig. 9b). No differences between any subgroups were detected on post hoc analyses of male or female mice. In the guanidine-soluble fraction from male mice, there was a trend towards a significant effect of APOE genotype, but not Cre genotype, on apoE protein level (Fig. 9g). In female mice, there was a significant effect of APOE genotype, but not Cre genotype, on apoE protein level (Fig. 9h). Post hoc analysis found no significant effect of Alb-Cre on guanidine-soluble apoE in either male (Fig. 9g) or female (Fig. 9h) mice.

Next, we analyzed total Aβ levels in APP/PS1/EKI Cre mice and their respective Cre−/− littermates. For these analyses, cortical samples from APP/PS1–21 mice with global deletion of murine Apoe (APP/PS1/EKO mice) were included for comparative purposes, and were not included in statistical analyses due to low n (n = 4, 3 males, 1 female). The cortices were sequentially homogenized in PBS and 5 M guanidine HCl buffer. Aβ and apoE levels were quantified by ELISA. a Levels of PBS-soluble apoE in cortex of male mice. There were no significant effects of apoE isoform (F2,50 = 3.040, p = 0.0568), Cre expression (F1,50 = 0.3673, p = 0.5472) or interaction (F2,50 = 0.1696, p = 0.8945). (n; APP/PS1/E2F: 8 Cre−/−; 12 Cre+/−; APP/PS1/E3F: Cre−/−; 12 Cre+/−; APP/PS1/E4F: 5 Cre−/−; 8 Cre+/−) b Levels of PBS-soluble apoE in cortex of female mice. There was no significant effect of apoE isoform (F2,42 = 0.1696, p = 0.8468), Cre expression (F1,42 = 0.02187, p = 0.8831) or interaction (F2,42 = 2.277, p = 0.1151). (n; APP/PS1/E2F: 5 Cre−/−; 8 Cre+/−; APP/PS1/E3F: 11 Cre−/−; 6 Cre+/−; APP/PS1/E4F: 11 Cre−/−; 7 Cre+/−) c Levels of PBS-soluble Aβ40 in the cortex of male mice. There was a significant effect of apoE isoform (F2,42 = 10.12, p = 0.0002), but no Cre expression (F1,42 = 0.022707, p = 0.8957) or interaction (F2,42 = 0.1413, p = 0.6650). (n; APP/PS1/E2F: 8 Cre−/−; 12 Cre+/−; APP/PS1/E3F: 10 Cre−/−; 12 Cre+/−; APP/PS1/E4F: 5 Cre−/−; 8 Cre+/−) d Levels of PBS-soluble Aβ42 in the cortex of female mice. There was a significant effect of apoE isoform (F2,42 = 3.495, p = 0.0394), but no Cre expression (F1,42 = 0.6344, p = 0.429) or interaction (F2,42 = 0.001, p = 0.97). (n; APP/PS1/E2F: 5 Cre−/−; 12 Cre+/−; APP/PS1/E3F: 11 Cre−/−; 12 Cre+/−; APP/PS1/E4F: 5 Cre−/−; 8 Cre+/−) e Levels of PBS-soluble Aβ40 in cortex of male mice. There was a significant effect of apoE isoform (F2,42 = 3.532, p = 0.0080), but no Cre expression (F1,42 = 0.7241, p = 0.4790) or interaction (F2,42 = 0.2245, p = 0.7999). (n; APP/PS1/E2F: 5 Cre−/−; 8 Cre+/−; APP/PS1/E3F: 11 Cre−/−; 6 Cre+/−; APP/PS1/E4F: 11 Cre−/−; 7 Cre+/−) f Levels of PBS-soluble Aβ42 in the cortex of female mice. There was a trend towards a significant effect of apoE isoform (F2,42 = 2.597, p = 0.0864), and no significant effect of Cre expression (F1,42 = 0.1586, p = 0.6925) or interaction (F2,42 = 0.0991, p = 0.97). (n; APP/PS1/E2F: 5 Cre−/−; 8 Cre+/−; APP/PS1/E3F: 11 Cre−/−; 6 Cre+/−; APP/PS1/E4F: 11 Cre−/−; 7 Cre+/−) g Levels of guanidine-soluble apoE in the cortex of male mice. There was a trend towards a significant effect of apoE isoform (F2,42 = 2.513, p = 0.0912), and no effect of Cre expression (F1,42 = 0.3746, p = 0.5479) or interaction (F2,42 = 0.001, p = 0.97). (n; APP/PS1/E2F: 8 Cre−/−; 12 Cre+/−; APP/PS1/E3F: 11 Cre−/−; 12 Cre+/−; APP/PS1/E4F: 5 Cre−/−; 8 Cre+/−) h Levels of guanidine-soluble apoE in the cortex of female mice. There was a significant effect of apoE isoform (F2,42 = 3.669, p = 0.0340), but no significant effect of Cre expression (F1,42 = 0.0003, p = 0.9857) or interaction (F2,42 = 0.0874, p = 0.7517). (n; APP/PS1/E2F: 5 Cre−/−; 8 Cre+/−; APP/PS1/E3F: 11 Cre−/−; 6 Cre+/−; APP/PS1/E4F: 11 Cre−/−; 7 Cre+/−) i Levels of guanidine-soluble Aβ40 in the cortex of female mice. There was a significant effect of apoE isoform (F2,42 = 7.273, p = 0.0017), but no Cre expression (F1,42 = 0.1102, p = 0.7414) or interaction (F2,42 = 2.333, p = 0.1079). (n; APP/PS1/E2F: 7 Cre−/−; 12 Cre+/−; APP/PS1/E3F: 11 Cre−/−; 12 Cre+/−; APP/PS1/E4F: 5 Cre−/−; 7 Cre+/−) j Levels of guanidine-soluble Aβ42 in the cortex of female mice. There was a significant effect of Cre expression (F1,42 = 0.1030, p = 0.7499) and no interaction (F2,42 = 0.0000, p = 0.997). (n; APP/PS1/E2F: 4 Cre−/−; 8 Cre+/−; APP/PS1/E3F: 11 Cre−/−; 6 Cre+/−; APP/PS1/E4F: 11 Cre−/−; 7 Cre+/−) k Levels of guanidine-soluble Aβ40 in the cortex of male mice. There was a significant effect of apoE isoform (F2,42 = 10.88, p = 0.0001), but no significant effect of Cre expression (F1,42 = 0.05731, p = 0.8118), and a trend towards a significant interaction (F2,42 = 0.925, p = 0.0631). (n; APP/PS1/E2F: 7 Cre−/−; 12 Cre+/−; APP/PS1/E3F: 11 Cre−/−; 12 Cre+/−; APP/PS1/E4F: 5 Cre−/−; 8 Cre+/−) l Levels of guanidine-soluble Aβ42 in the cortex of male mice. There was a significant effect of apoE isoform (F2,42 = 16.32, p < 0.0001), but no Cre expression (F1,42 = 0.4266, p = 0.5173) and no interaction (F2,42 = 1.722, p = 0.1914). (n; APP/PS1/E2F: 4 Cre−/−; 8 Cre+/−; APP/PS1/E3F: 11 Cre−/−; 6 Cre+/−; APP/PS1/E4F: 11 Cre−/−; 7 Cre+/−). Data analyzed by 2 way ANOVA. Data from APP/PS1/EKO mice (3 males, 1 female) are also plotted, but were not included in statistical analysis.
Together, these results suggest that, while depletion of human APOE expression in hepatocytes led to a marked lowering of plasma apoE with some changes in plasma lipid composition, there were no significant effects on cerebral Aβ accumulation.

Discussion
APOE is the strongest genetic risk factor for late-onset AD and intensive research efforts have led to several important insights regarding apoE and its role in AD. Nevertheless, cell-type specific roles for APOE isoform expression, secretion, and lipiddation in neurodegenerative disease remain poorly understood. In an effort to help to begin to answer these and other questions, we created a new generation of human APOE-expressing mice to study cell-specific processes. Specifically, we generated three separate lines of APOE-KI mice, each carrying one of the three most common variants of the human APOE gene. The presence of loxP site on either side of the human gene sequence allow for cell-type-specific manipulation of APOE expression through the Cre-loxP system. Here, we characterized the newly created mice in terms of CNS as well as peripheral expression. We also qualitatively compared apoE particles isolated from astrocytes and microglia, and found the latter to produce significantly smaller lipid-containing particles. We took a step further to validate the functionality of the loxP sites by specifically ablating hepatocyte expression of APOE, effectively eliminating the majority of apoE protein in the plasma. We found this virtual absence of apoE in the plasma to cause significant alterations in the plasma lipid profile without significantly altering cerebral amyloid plaque accumulation in a model of Aβ-driven amyloidosis.

Our targeting construct retained the natural genetic context surrounding the human exon sequence, including endogenous regulatory elements such as enhancers. Thus, we expected the tissue-specific expression of the human APOE gene to closely parallel that of the endogenous mouse ApoE gene. Our APOE gene expression and protein analysis in the brain and plasma showed a similar expression level to wild type C57/BL6 mice and other APOE knock-in mice [1, 6, 43, 65]. Interestingly, we detected a subtle but statistically significant isoform-dependent difference in APOE mRNA levels in APOE-KI mice, where higher mRNA levels were detected in E4F mice relative to E2F or E3F mice in the hippocampus and lower levels of mRNA were detected in the brain hemisphere of E2F mice compared to E3F or E4F mice. These different mRNA levels could arise from compensatory changes in APOE transcription in response to isoform-dependent differences in protein stability or subtle differences in mRNA stability. Other than our new model, one other APOE knock-in model with floxed APOE alleles has been described [9]. In one experiment, apoE4 reduction in adult hippocampal astrocytes using an AAV-Cre vector resulted in a 50% decrease in insoluble Aβ42 in PDAPP mice [9]. Future studies using floxed allele APOE KI mice can test the temporal and cell-type-specific effects of disrupting APOE expression on Aβ and tau pathology using a variety of inducible Cre mouse strains.

At 3 months of age, there was significantly less PBS-soluble apoE in the cortex of E4F mice compared to those from E2F or E3F mice at the same age, consistent with previously described findings in human and other APOE transgenic and knock-in mouse models [6, 37–44]. Concordantly, the level of apoE2 protein is the highest compared with other apoE isoforms in the CSF [6, 66], interstitial fluid (ISF) [67], brain parenchyma [68], and plasma [69–71]. The protein level of apoE2 is generally higher both in brain and plasma, likely due to decreased strongly reduced binding affinity to the LDL receptor [72]. Some studies report apoE4 is more susceptible to proteolysis compared to the other major isoforms of APOE [73, 74], and other studies have demonstrated the presence of apoE4 fragments (14–20 kDa) in AD brains [75, 76]. Structural differences between apoE3 and apoE4, particularly at the hinge region between the N- and C-terminal domains, may explain their different susceptibility to proteolytic degradation as well as lipid binding affinity [77]. In spite of these numerous observations, the exact nature of the protease responsible for apoE4 cleavage is unknown, although several candidates have been reported including cathepsin D [78], aspartic proteases [79], and a chymotrypsin-like protease [73]. A prior study using APOE-TR mice reported differences in apoE protein levels only in the hippocampus and cortex, regions that are susceptible to neurodegeneration in AD brains [43]. Significant differences in protein levels among apoE isoforms (apoE4 < apoE3 < apoE2) were also observed in the CSF and brain homogenates of APOE-TR mice when crossed to PDAPP mice [6]. It will be important in future studies to assess both CSF and brain levels of apoE in our new model in multiple brain regions as well as in all relevant cell types. Importantly, these results should be complemented with similar studies in human.

In regards to the effects of apoE isoforms in our new model on Aβ aggregation and accumulation in the brain, we found very similar findings that we described previously in APP/PS1–21 mice crossed to another APOE knock-in model [8, 10]. Namely, the presence of apoE4 in the brain resulted in significantly greater Aβ deposition than apoE3, similar to what is seen in humans [7, 80]. However, when different APP transgenic mouse strains were crossed with other APOE knock-in mice, apoE2-expressing mice generally exhibited less Aβ deposition than those expressing apoE3 or apoE4 [5, 6, 9], also
similar to what is seen in humans. Crossing of APP/PS1–21 mice to our new APOE-KI model results in similar levels of Aβ accumulation in those expressing apoE2 and apoE4. Interestingly, crossing of another APOE knock-in model (APOE-TR mice) with APP/PS1–21 mice also resulted in similar levels of Aβ deposition in apoE2- and apoE4-expressing mice (Additional file 2: Figure S2a and S2c). The effect of sex on Aβ pathology in our new EKI models are intriguing, as we did not detect an effect of sex on Aβ pathology when the APOE-TR mice were crossed to the same strain of APP/PS1–21 mice (Additional file 2: Figure S2b and S2d). In another APP/PS1 transgenic model, 5XFAD mice, apoE2-expressing mice were also found to have similar Aβ deposition to those expressing apoE3 or apoE4 in the subiculum [43]. In aggressive amyloidogenic models such as APP/PS1–21 and 5XFAD, due to the particular APP and PS1 mutations present, there is a much higher ratio of Aβ42 to other Aβ species than in many less aggressive APP transgenic mice [50]. Since Aβ42 is more aggregation-prone than other Aβ species [81], this difference might abrogate the effect of apoE2 on lowering Aβ pathology compared to other models and in humans. Further studies with our new model can test this hypothesis in other APP models such as APP knock-in mice or APP transgenic mice.

Outside of the brain, apoE is synthesized in multiple other sites, including the liver, spleen, adrenal gland, lung, testis, and ovary [82–84]. Total knock-out of Apoe results in severe hypercholesterolemia with accelerated atherosclerosis in the periphery [85, 86], accompanied in some [21] but not all studies [87] by synaptic loss and cognitive dysfunction. Due to the nature of a global knock-out, it was difficult to assess in the latter finding whether lack of apoE in the brain, in the periphery, or both, was responsible for the aforementioned phenotype. Given these outstanding questions, we tested whether specific ablation of a large source of peripheral apoE could modulate cerebral Aβ pathology. Indeed, virtual ablation of plasma apoE using an Alb-Cre line did not result in any change in cerebral apoE levels, which is consistent with previous reports on the inability of peripheral apoE to cross the BBB [20] or the blood-CSF barrier [88]. It is worth noting that hepatocytes are not the sole source of apoE in the periphery, as peripheral macrophages [82] and adipocytes [89] are known to contribute to the pool of apoE in the plasma. Accordingly, we did detect a non-negligible amount of apoE protein in plasma from APP/PS1/E2Fcre mice, likely due to apoE2’s low affinity for the LDL receptor [90, 91] that results in slower clearance from the plasma. The lack of detection in APP/PS1/E3Fcre and APP/PS1/E4Fcre was perhaps due to a combination of lower protein levels and over-dilution that put the concentration outside of the assay’s detection limits. Our histological and biochemical analyses of cerebral Aβ pathology did not reveal a significant effect of knocking out liver-derived apoE at 4 months of age. Additionally, we failed to detect any changes in the degree of astrogliosis or microgliosis immediately surrounding the plaques between APP/PS1/ EKI Cre mice and their Cre +/– littermates (data not shown). Altogether, our data suggest CNS- and peripherally-derived apoE exist in distinct pools that are independent from one another, as has been suggested in human studies [92]. However, we cannot rule out other hypotheses that would otherwise explain the lack of any appreciable effect on Aβ in APP/PS1/EKI Cre mice, such as an unknown adaptive response that masked the contribution of hepatic apoE to brain Aβ pathology.

Alternatively, it is possible that apoE from one pool can indirectly exert an effect on the other side of the BBB. For example, a recent study showed that restoration of peripheral Apoe expression led to a partial rescue of cognitive phenotypes in mice lacking apoE in the brain [28]. These findings suggest a dual mechanism by which apoE deficiency causes behavioral deficits, and that peripherally derived apoE may influence neuronal function through an indirect mechanism, such as vascular dysfunction secondary to dyslipidemia or via effects on brain endothelial cells that make up the BBB. In support of this latter hypothesis, prior studies found the BBB in EKO mice to be severely compromised [24, 93], and the severity (or permeability of the BBB) also increased with age [93]. Thus, it is conceivable that an effect of hepatocyte-derived apoE on brain Aβ pathology may be observed in aged APP/PS1/EKI Cre mice, or if conditional knock-out of hepatic apoE occurs at a later stage of Aβ pathology. Such an effect may be modulated by apoE’s effects on cerebrovascular function and/or through its ability to cross a leaky BBB. Some researchers have proposed a two-hit vascular hypothesis of AD cerebrovascular damage, where hit 1 is an initial insult on the BBB itself that is sufficient to initiate neuronal injury and neurodegeneration, but can also promote accumulation of Aβ in the brain through defects in clearance of Aβ through the BBB (reviewed extensively in [94, 95]).

BBB dysfunction is a common co-occurrence and may directly contribute to neurodegeneration and cognitive decline in AD [94, 95]. In support of this latter hypothesis, data obtained from an older APOE knock-in model also support an isoform-dependent effect of apoE on BBB integrity, where EKO and apoE4-expressing mice developed vascular defects before neuronal and synaptic changes occur [25]. A more recent study showed apoE4-expressing mice to have impaired spontaneous BBB repair following traumatic brain injury (TBI) compared to apoE2- or apoE3-expressing mice [96]. Both studies explored various
mechanisms that could explain for the negative influence of apoE4 on the BBB integrity, all of which involved changes in astrocytes and pericytes located in the neurovascular unit. However, it is theoretically possible that peripherally derived apoE species can somehow contribute to this process, especially with the leakiness of the BBB in early stages of TBI and later stages of AD. As there are several sources of apoE in the periphery, they could differentially affect BBB homeostasis. Intriguingly, a prior study utilized bone marrow transplant to show that BBB homeostasis depends on equal contributions from tissue and blood cell derived apoE, but lack of *Apoe* expression in bone marrow-derived cells alone was enough to significantly increase the BBB permeability [93]. These findings underscore the putative role for leukocytes (e.g. macrophages) in BBB maintenance. It remains elusive how apoE from bone marrow-derived cells may contribute to the integrity of the BBB, the dysfunction of which had been proposed to contribute to neurodegeneration in AD [95]. In this context, it would be interesting to investigate whether restoration of *ApoE-e4* expression in peripheral macrophages could rescue BBB defects in EKO mice, and how that compares to restoration of *ApoE-e2* or *ApoE-e3* expression.

It is our hope that these new *ApoE-KI* mice will facilitate studies into apoE physiology and AD pathogenesis. It is also important, however, to acknowledge their limitations. While the *ApoE-KI* mice harbor the human gene sequence, they retain the regulatory elements found in mice. Considerable species differences between rodents and humans exist and might challenge our ability to generate findings that are all relevant and directly translatable to humans from studies in mice and rats. Apparent differences in physiological function and metabolism, such as lipid metabolism and immune response between humans and rodents might preclude some discoveries that are relevant to disease mechanism. For example, apoB is a ligand for the LDLR along with apoE in humans, albeit with a lower affinity than apoE. Hepatic-derived apoB is secreted as apoB100 (a full length protein) and contains the LDLR binding domain. However, a large portion of hepatically derived apoB in mice is truncated (apoB48) and does not contain the LDLR domain. Wild-type mouse VLDL and IDL contain roughly equal portions of apoB48 and apoB100, and this leads to a compromised compensatory mechanism in the absence of apoE, leading to severe hypercholesterolemia in *ApoE* knock-out mice [86, 97]. This latter example highlights the need to address these and other caveats when interpreting rodent studies, especially in those where such physiologic differences might confound some findings.

**Conclusions**

We employed a targeted gene replacement strategy to generate mouse lines that express the three most common alleles of the human *APOE* gene at physiological levels. We fully characterized all three lines with respect to their apoE levels in the CNS as well as the plasma, from early life to adulthood. We partially recapitulated the isoform-dependent effect of *APOE* on Aβ accumulation in a model of amyloidosis. Furthermore, we also validated the functionality of the loxP sites in facilitating constitutive, tissue-specific, knock-out of *APOE* through liver-specific expression of Cre-recombinase. Lastly, we also investigated the effects of knocking out liver-derived apoE on plasma lipid profiles and Aβ deposition in the brain. Moving forward, these mice should prove an invaluable asset for further studies on the physiological and pathophysiological roles of *APOE*, especially the role of apoE isoforms in specific cell types and different organs in the context of AD and other neurodegenerative diseases.

**Additional files**

*Additional file 1: Figure S1.* Replacement of the mouse *ApoE* gene with the human *ApoE* gene in *ApoE-KI* mice. a The specific sequence of the genomic region surrounding the translation initiation codon of the human and mouse *ApoE* gene, located on exon 2, is shown. The mouse and human sequences are aligned for comparative purposes. b–d Targeting vectors for the E2 and E4 alleles have identical sequence other than their respective SNPs at positions 130 and 176, both located on exon 4 ([Cys130, Arg176] for *ApoE-e2* (b), [Cys130, Arg176] for *ApoE-e3* (c), and [Arg130, Arg176] for *ApoE-e4* (d)). The red arrowheads identify the SNPs at positions 130 and 176 in each of the allele sequences. e, f *ApoE* mRNA levels in the hippocampus (e), and hemisphere (f) of *ApoE-KI* mice were analyzed at 3 months of age (*p* = 0.0169, *F* = 5.843 and *p* = 0.0025, *F* = 10.88, respectively). g Cortex-derived brain homogenates from 3-month-old *ApoE-KI* mice were subjected to western blot analysis for apoE using antibody HJ15.3. White arrowhead = sialylated apoE (MW ~ 35.3 kDa). Black arrow = non-sialylated apoE (MW ~ 33.6 kDa). *p* < 0.05, **p** < 0.01, ***p*** < 0.001. A one-way ANOVA was used to assess significance between more than two groups, and Bonferroni’s post-hoc test was used to test for differences between each of the groups. All values are reported as mean ± SEM. *N* = 5 per genotype for ELISA analysis, with approximately equal numbers of males and females. (TIF 3191 kb)

*Additional file 2: Figure S2.* ApoE isoforms differentially influence Aβ plaque deposition in APP/PS1/APOE-TR mice. a Brain sections from 3-month-old APP/PS1/APOE-TR mice were immunostained with anti-Aβ antibody 3D6 and the extent of Aβ deposition in the cortex quantified. Two-way ANOVA analysis found a significant effect of apoE isoform (*F* 3,38 = 10.13, *p* = 0.0003) but no significant effect of sex (*F* 1,38 = 0.001177, *p* = 0.9728), and no interaction (*F* 3,36 = 0.06101, *p* = 0.9409). Post hoc analysis comparing apoE isoform within each sex found a statistically significant increase in Aβ deposition in male apoE2~ (p = 0.0010) and apoE4~ expressing (*p* = 0.0430) mice compared to apoE3, APP/PS1/E2 = 8 males, 9 female; APP/PS1/E3 = 11 males, 4 females; APP/PS1/E4 = 7 males, 5 females. b Since there was no effect of sex on Aβ staining, data for both males and females were pooled together. A one-way ANOVA was performed (*F* = 11.92, *p* < 0.0001), followed by Tukey’s post hoc test for multiple comparisons. c Brain sections from 4-month-old APP/PS1/APOE-TR mice were stained with X-34 dye that recognizes only fibrillar plaques and the cortical area stained by X-34 was quantified. There was a significant effect of genotype (*F* 2,37 = 8.701, *p* = 0.0008) but no significant effect of sex.
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Authors’ contributions
T-PVH, CW, JDU, and DMH conceived the project and designed the experiments. T-PVH and CW performed most of the experiments, assisted by ACT, GTT, TEM, CMF, MBF, RS, MM, RET, and JDU. All authors read and commented on the manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Not applicable.

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Abbreviations
AD: Alzheimer disease; ApoE: Apolipoprotein E; Aβ: Amyloid-β; BAC: Bacterial artificial chromosome; EKO: ApoE knock-out; GFAP: Glial fibrillary acidic protein; HDL: High density lipoprotein; IBA1: Ionized calcium binding adaptor molecule 1; LDLR: Low density lipoprotein receptor; NDGGE: Non-denaturing gradient gel electrophoresis; SEM: Standard error of the mean; TC: Total cholesterol

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