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Peripheral apolipoprotein E proteins and their binding to LRP1 antagonize Alzheimer's disease pathogenesis in the brain during peripheral chronic inflammation

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Abstract

C-reactive protein (CRP) impacts apolipoprotein E4 (ApoE4) allele to increase Alzheimer's disease (AD) risk. However, it is unclear how the ApoE protein and its binding to LRP1 are involved. We found that ApoE2 carriers had the highest but ApoE4 carriers had the lowest concentrations of blood ApoE in both humans and mice; blood ApoE concentration was negatively associated with AD risk. Elevation of peripheral monomeric CRP (mCRP) reduced the expression of ApoE in ApoE2 mice, while it decreased ApoE-LRP1 binding in the brains of ApoE4 mice that was characterized by Proximity Ligation Assay. Both serum ApoE and brain ApoE-LRP1 binding were positively associated with the expression of pericytes that disappeared after mCRP treatment, and negatively associated with brain tauopathy and neuroinflammation in the presence of mCRP. In ApoE$^{-/-}$ mice, mCRP reduced the brain expression levels of synaptophysin and PSD95 and the positive relationship between ApoE-LRP1 binding and synaptophysin or PSD95 expression disappeared. Our study suggests that blood ApoE protects against AD pathogenesis by binding to LRP1 during peripheral chronic inflammation.

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CRediT authorship contribution statement

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Keywords

ApoE; mCRP; Alzheimer's disease; LRP1; pericytes; synaptic biomarkers

1. Introduction

Apolipoprotein E4 (ApoE4) is the major genetic risk factor for late-onset Alzheimer's disease (AD) (Strittmatter et al., 1993). However, not all ApoE4 carriers develop AD, even among those who exceed 90 years of age (Tanzi, 1999). It has been reported that the ApoE isoform (ApoE2 > ApoE3 > ApoE4) affects the clearance of amyloid-β (Aβ), a major component of AD pathology, from the brain (Castellano et al., 2011). In addition, ApoE bound to Aβ is internalized through the cell surface ApoE receptor low-density lipoprotein receptor-related protein (LRP)1 (LaDu et al., 1994). LRP1 also has effects on Aβ clearance in vascular smooth muscle cells and transport across the blood brain barrier in APP/PS1 mice (Kanekiyo et al., 2012; Pflanzner et al., 2011). All these findings suggest that brain ApoE protein and ApoE-LRP1 binding play some roles in AD pathogenesis; however, it is unclear whether peripheral ApoE influences AD pathology formation in the brain through LRP1 binding. Since LRP1 can modulate immune responses via proinflammatory cytokines and microglial activation in the brain (Yang et al., 2016), it is probable that peripheral ApoE and ApoE-LRP1 binding are involved in brain AD pathogenesis by regulating inflammatory processes.

Multiple studies have reported that circulating ApoE levels determined by ApoE genotypes (Mooijaart et al., 2006; Marais, 2019). Importantly, using the ApoE target replacement (TR) mouse models, the human E4 allele impacts the low levels of ApoE protein both in blood and brain, and the dominant negative effect on cognitive ability (Johnson et al., 2014; Lane-Donovan and Herz, 2016). In addition, the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes cognitive disability only in ApoE4, but not in ApoE3, mice (Torres et al. 2020). Our study used data from the Framingham Heart Study (FHS) offspring cohort and found that chronically elevated C-reactive protein (CRP) levels in blood are associated with an increased risk of AD in ApoE4 carriers but not in ApoE3 or ApoE2 carriers (Tao et al., 2018). ApoE protein, like CRP, is mainly produced by the liver and is released into the blood when systematic inflammation occurs. We aimed to study whether peripheral ApoE and its binding to LRP1 protect against AD pathogenesis in the brain when peripheral CRP levels are elevated. As systemic chronic low-grade inflammation is a modifiable and potentially treatable medical condition, identifying and characterizing whether ApoE and CRP play interacting roles leading to AD in ApoE4 carriers is useful for prevention of and intervention in the disease.

CRP is a protein that plays a role in the immune response to toxins or injuries in systemic inflammation and is used to monitor peripheral chronic low-grade inflammation in clinical practice (Stephensen and Gildengorin, 2000). There are two forms of CRP (Slevin and Krupinski, 2009): 1) native CRP (pCRP) is a pentameric oligoprotein and an acute phase reactant that is produced during active inflammatory reactions; and 2) monomeric CRP (mCRP), or free subunits of pCRP, is produced during the acute phase and more so during

a chronic phase by the irreversible dissociation of pCRP (Potempa et al., 2015). mCRP has a much lower aqueous solubility and can cause tissue damage (Caprio et al., 2018). CRP levels increase with age (Stephensen and Gildengorin, 2000, Tang et al., 2017), probably because infections of the respiratory, gastrointestinal, and urinary tract systems are more likely to occur in the elderly and induce chronic low-grade inflammation. mCRP has been shown to play a role in the pathogenesis of cardiovascular diseases (Wang et al., 2015), age-related macular degeneration (Chirco et al., 2016), and poststroke inflammation (Slevin et al., 2010). In one study, mCRP was directly injected into the hippocampus of an AD mouse model, 3×Tg, and it was found that mCRP injection increased the severity of AD pathology in the brain (Slevin et al., 2015). In this study, we hypothesized that elevation of peripheral mCRP levels induces/enhances AD pathology in the brain, whereas blood ApoE and ApoE-LRP1 binding antagonize mCRP to promote AD pathogenesis in the brain during peripheral chronic low-grade inflammation. We used human data as well as different ApoE knock-in and ApoE$^{-/-}$ mice to characterize the relationship between peripheral ApoE, ApoE-LRP1 binding, and AD pathogenesis in the brain in the absence and presence of elevated blood mCRP levels.

2. Material and Methods

2.2 Framingham Heart Study

This study used the existing data of the Framingham Heart Study (FHS). FHS is a single-site, community-based, prospective cohort study in Framingham, MA. The design and selection criteria of the Framingham Heart Study offspring cohort (Gen 2) have been previously described (Kannel et al., 1979). The source population for this study was 2723 FHS offspring participants at Exam 5 (exam date: 1991 to 1995) 1) who were aged 28 years or older, 2) who underwent ApoE genotyping and baseline plasma ApoE protein concentration measurement during that exam and 3) for whom there were data on AD development. The incidence of AD (follow-up time: median 23.1 (IQR 19.2, 24.4) years) was used as the outcome for analyses. Informed consent was obtained from all study participants and the study protocol was approved by the Institutional Review Board of Boston University Medical Campus.

2.1. Mice and experimental treatments

All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Boston University Animal Care and Use Committee (PROTO201800192). Because that the APOE-ε4 risk for AD is greater in women than men (Riedel et al., 2016), we chose female ApoE knock-in mice for the experiments. Female ApoE genetic knock-in mice were purchased from Taconic Biosciences, Inc. (ApoE2: #1547-F, ApoE3: #1548-F, ApoE4: #1549-F, Rensselaer, NY, USA). The endogenous mouse ApoE genes in the genetic knock-in mice were replaced with human ApoE genes (either the ApoE2, ApoE3 or ApoE4 allele). ApoE knockout mice (#ApoE-F) were purchased from Taconic, Inc., for use as a control group.

All mice were maintained in microisolator housing in the animal facility at Boston University School of Medicine. mCRP was dissolved in phosphate-buffered saline (PBS),

and vehicle-treated mice were injected with PBS only as a control. mCRP (200 μg/kg) vs. PBS was injected intraperitoneally (i.p.) into female ApoE mice aged 10 months three days per week (Monday, Wednesday, and Friday) for 6 weeks (n = 12–14 ApoE knock-in mice, n = 7 ApoE knockout mice).

2.2. Enzyme-linked immunosorbent assay

After sacrificing the mice, each brain from the mice was divided into right and left halves. One half was frozen in −80 degrees for ELISA and the other half was fixed for IHC. For ELISA, protein was extracted by homogenization and ultrasonication. Homogenate was centrifuged at 5000 g for 5 minutes and collect supernatant. For immunohistochemistry, brain was post-fixed in 4% paraformaldehyde for 2 days and treated with 30% sucrose in PBS for 2 days. Brain cut for floating serial coronal cryosections (30 μm) and stored at 4°C before use. Human ApoE protein concentrations in the serum and brain were tested by the ApoE ELISA Kit (#LS-F3543, LifeSpan BioSciences, Seattle, WA, USA). This kit detects the ApoE proteins regardless of different isoforms. We used knock-in mice with different isoforms of human genes; thus, we detected the total ApoE levels in serum and brain. Serum was obtained from blood collected by cardiac puncture, and brain protein samples were extracted from the hippocampus. The blood samples were centrifuged for 15 minutes at 3000 rpm. Serum and brain samples and reagent preparation were performed for the measurement of ApoE concentrations according to the LifeSpan user manual, and the absorbance was measured at 450 nm with a microplate reader (BioTek instruments, Winooski, VT, USA). The mouse Aβ42 level in the hippocampal extracts was measured using an Amyloid Beta 42 Mouse ELISA Kit (Invitrogen, # KMB3441). All assays were performed in duplicate, and the average of two values was used for further analysis.

2.3. Immunofluorescence characterization

Immunofluorescence was used to characterize the pathology in the mouse brain (n = 12–14 for each condition). Mouse brains were collected after PBS perfusion, the half of brain postfixed in 4% paraformaldehyde for 48 hours, and incubated in 30% sucrose in PBS at 4 °C. The free-floating staining method was used to stain coronal cryosections (30 μm thick). The brain sections were preincubated in blocking solution containing 5% [vol/vol] horse serum (Sigma–Aldrich, St. Louis, MO, USA) and 1% bovine serum albumin in 1× Tris-buffered saline with Tween 20 for 2 hours at room temperature. The slides were incubated individually with primary antibodies overnight. Rabbit monoclonal antibodies against ApoE (#701241, Invitrogen, Carlsbad, CA, USA) and mouse monoclonal low-density lipoprotein receptor-related protein 1 (#SC-57353, Santa Cruz Biotechnology, Dallas, TX, USA) antibodies were used to detect ApoE and its receptor. Phosphorylated tau was detected with an anti-PHF1 antibody obtained from Dr. Wolozin at Boston University School of Medicine. Microglia were detected with an anti-CD68 antibody (#MCA1957GA, Bio–Rad Laboratories, Hercules, CA, USA), and astrocytes in the brain were detected with an anti-GFAP antibody (#14-9892-82, Fisher Scientific, Hampton, NH, USA). Pericytes at the blood–brain barrier were detected with an anti-PDGFRβ antibody (#3169S, Cell Signaling Technology, Danvers, MA, USA). Synaptophysin (#SC-17750, Santa Cruz Biotechnology) was used as a presynaptic marker, and PSD95 (#3450S, Cell Signaling) was used as a postsynaptic marker. The brain slices were then stained with

secondary antibodies conjugated to Alexa Fluor 488, 594, or 549 (1:700, Thermo Fisher Scientific, Carlsbad, CA, USA) for 1 hour at room temperature. The sections were mounted with ProLong Gold antifade reagent with DAPI for nuclear staining (#P36935, Thermo Fisher Scientific). The stained brains were observed under a fluorescence microscopy (Carl Zeiss, Germany).

ImageJ software was used to analyze the immunostaining results by measuring the total intensity after adjusting the threshold. The data from two independent investigators who were blinded to the treatment groups were pooled and averaged.

2.4. Proximity ligation assay (PLA)

A proximity ligation assay (PLA) was applied to investigate protein–protein interactions (Alam, 2018; Zieba et al., 2018). Brightfield PLA was performed according to the manufacturer's protocol (#DUO92001 and #DUO92005, Sigma–Aldrich) with minor modifications. Brain sections from ApoE genotype mice were fixed with 4% paraformaldehyde and washed with PBS. The sections were blocked with Duolink Blocking Solution for 1 hour at 37 °C, and the slices were subsequently incubated with ApoE and LRP1 primary antibodies overnight in a humid chamber at 4 °C. The following day, the slices were incubated with the secondary PLA probes anti-mouse PLUS and anti-rabbit MINUS in a preheated humid chamber for 1 hour at 37 °C. After washing, the sections were ligated (#DUO92007, Sigma–Aldrich) for 30 minutes and amplified with polymerase for 100 minutes at 37 °C. The sections were visualized by the addition of Duolink In Situ Mounting Medium with DAPI. The PLA signals were detected using fluorescence microscopy (ZEISS Axio) and manually assessed using ImageJ software.

2.5. Statistical analysis of the FHS data

Analyses were performed using Statistical Analyses System software version 9.3 (SAS Institute, Cary, NC) and the R Language and Environment for Statistical Computing (version 3.6.3). The statistician who conducted the data analysis was not involved in any measurement in this study. We performed univariate analyses to describe the baseline characteristics of the final sample population, which was stratified by ApoE genotype (ApoE2 = ApoE2/2 or 2/3; ApoE3 = ApoE3/3; ApoE4 = ApoE3/4 or 4/4). The mean ± SD was determined, and ANOVA was conducted for variables with normal distribution. Mann–Whitney tests using the medians (Q1, Q3) were performed for variables with a skewed distribution, and chi-square tests using numbers (%) were used for categorical variables.

The sample size excluded those FHS participants who had dementia (n = 62), missed CRP data (n=343) or education (n = 440) at baseline. Kaplan–Meier survival analyses were performed to compare the onset of AD in the follow-up (follow-up time: median 23.1 (IQR 19.2, 24.4) years) among ApoE quantiles group, Q1: [0, 6.74], Q2: [6.74, 9.23), Q3: [9.23, 12.33), and Q4: [12.33, 33.06]. Further, Cox proportional hazards models were used to examine ApoE protein quantiles or concentration (log transformed) and the development of AD in the following three models: Model 1: Unadjusted association between AD onset and ApoE protein levels. Model 2: Adjusting for age and sex. Model 3: Model 2 plus CRP (log

transformed) and education variable. Stratification based on the ApoE genotype was applied. In all models, a significance level of $\alpha = 0.05$ of two-tailed test was used.

2.7. Statistical analyses of mouse data

The mouse data in the experiments were analyzed by multifactorial analysis of variance (ANOVA) followed by Tukey's post hoc test and Student's *t* test using GraphPad Prism 8.0 software (San Diego, CA, USA). The association between two factors was analyzed by Pearson's correlation using GraphPad Prism 8.0 software. A *p* value less than 0.05 was considered statistically significant in all experiments.

3. Results

3.1. Blood ApoE protein decreases AD risk in humans

First, we used human samples from the FHS to study the relationship between the protein concentration of plasma ApoE and AD risk (n = 2723). Fig. 1A shows that ApoE2 carriers had the highest average protein concentration of plasma ApoE, followed by ApoE3 carriers and then ApoE4 carriers (mean ± SD [mg/dL]: 14.2 ± 5.4 vs. 10.1 ± 4.4 vs. 7.6 ± 3.3, $p < 0.0001$). Fig. 1B shows that the average serum ApoE concentration significantly decreased as age increased in ApoE4 carriers only. Based on plasma ApoE concentrations, we divided the human samples into four quartiles (Q1, Q2, Q3 and Q4) (Fig. 1C). Using the log-rank test, we found that individuals in Q4, which comprised those with the highest protein concentrations of plasma ApoE, had the lowest risk of AD followed by those in Q3 and Q2; however, those with the lowest protein concentration of serum ApoE (Q1) had a higher of AD ($p = 0.03$).

We conducted Cox proportional hazards analysis and found that after adjusting for age, sex, education and serum CRP levels, high serum ApoE quartiles remained associated with decreased AD risk [hazard ratio 0.48 (95% CI 0.29–0.80), $p < 0.01$] (Supplementary Table 1). After ApoE4 genotype was added to the model, the impact of ApoE protein centration (Log transformed) was less significant for AD onset (Supplement Table 2). In order to study protein concentration vs. protein structure in ApoE protein to affect AD risk, we further stratified the FHS participants into those ApoE4 noncarriers and ApoE4 carriers and conducted Cox proportional hazards analysis (Supplement Table 2). Interestingly, ApoE protein concentration was associated with decreased AD risk only in ApoE4 noncarriers, but not in ApoE4 carriers, suggesting that both protein concentration and protein structure are critical for blood ApoE protein to protect brain from AD pathogenesis during peripheral inflammation.

3.2. Correlations of blood ApoE concentration with the brain levels of ApoE and LRP1 and ApoE-LRP1 binding

We next used knock-in mice expressing the human ApoE gene in parallel with human data. We measured ApoE levels in the sera and brains of mice with different ApoE genotypes. Consistent with the human findings (Fig. 1), ApoE2 mice also had a higher protein concentration of serum ApoE than ApoE3 ($p < 0.0001$) and ApoE4 ($p < 0.0001$) mice after PBS treatment. After intermittent intraperitoneal mCRP inject, ApoE2 mice still

had a higher protein concentration of serum ApoE than ApoE3 ($p = 0.0053$) and ApoE4 ($p < 0.0001$) mice (Fig. 2A). Additionally, regardless of the treatment (i.p. PBS or mCRP), the protein levels of ApoE were higher in the brains of ApoE2 mice than in those of ApoE3 ($p < 0.0001$) and ApoE4 ($p < 0.0001$), as determined by both immunostaining (Fig. 2B) and ELISA (Fig. 2C). Elevation of peripheral mCRP levels lowered the ApoE concentrations of protein in both the serum (21% reduction, Fig. 2A; $p = 0.0097$) and the brain in ApoE2 mice but not in ApoE3 or ApoE4 mice, as assessed by immunostaining (54% reduction, Fig. 2B; $p = 0.0011$) and ELISA (24% reduction, Fig. 2C; $p = 0.0055$). We found that the serum ApoE protein level was positively correlated with the brain ApoE protein level both in the absence (r = 0.5219, $p = 0.0004$) and presence (r = 0.5736, $p = 0.0002$) of intraperitoneal mCRP injection (Fig. 2D).

We then investigated the brain expression of LRP1, which is a main receptor of ApoE in vascular smooth muscle cells and in neurons in the brain (Kanekiyo et al., 2013). The expression level of LRP1 in the hippocampal CA3 region was higher in ApoE2 mice than in ApoE$^{-/-}$ ($p = 0.0118$), ApoE3 ($p = 0.0037$) and ApoE4 ($p = 0.0005$) mice after intraperitoneal PBS injection; similar findings were observed after intraperitoneal mCRP injection (Fig. 2E). Serum ApoE expression was significantly positively correlated with the brain expression of LRP1 after intraperitoneal PBS injection (r = 0.5642, $p = 0.0002$); however, after intraperitoneal mCRP injection, this positive correlation between serum ApoE expression and brain LRP1 levels disappeared (Fig. 2F).

Next, we examined the interaction between ApoE and LRP1 protein in the brain using a PLA immunoassay. ApoE2 mice exhibited a higher degree of ApoE-LRP1 binding in the hippocampal CA3 region than ApoE3 ($p < 0.0001$) and ApoE4 ($p < 0.0001$) mice regardless of treatment (PBS or mCRP) (Fig. 2G). Consistently, using immunostaining, we found that compared to those of mice with other ApoE genotypes, the brains of ApoE2 mice exhibited increased coexpression of ApoE and LRP1 (Supplementary Figure 1). Interestingly, using the PLA assay, we found that elevation of peripheral mCRP levels significantly 38% decreased ApoE-LRP1 binding in the brains of ApoE4 mice only ($p = 0.0296$, Fig. 2G). Regardless of the elevation of peripheral mCRP levels, serum ApoE expression still had a significant positive correlation with ApoE-LRP1 binding in the brain (Fig. 2H). The binding of rabbit IgG and LRP1 did not change in any of the ApoE groups (Supplementary Figure 2). The finding suggested that binding affinity of ApoE and LRP1 was affected by both their concentration and protein structure. Taken together, these results demonstrated that both the expression of ApoE and LRP1 and ApoE-LRP1 binding in the brain followed a pattern of ApoE2>ApoE3>ApoE4 regardless of whether peripheral mCRP levels were elevation; however, mCRP significantly reduced direct ApoE-LRP1 binding in the brains of ApoE4 mice only.

3.3. Changes in the brain induced by elevation of peripheral mCRP levels across different ApoE genotypes

Since peripheral mCRP is exposed to the blood–brain barrier (BBB) and the BBB has been shown to be damaged in AD (Bergers and Song, 2005, Beishon & Panerai, 2021; Hussain et al., 2021; Li et al., 2021), we first examined the influences of peripheral mCRP on the

expression of the pericyte biomarker PDGFRβ in the brain across different ApoE genotypes. After intraperitoneal PBS injection, ApoE2 mice had higher brain expression of PDGFRβ than ApoE$^{-/-}$ ($p = 0.0089$) and ApoE4 ($p = 0.0030$) mice (Fig. 3A and Supplement Fig. 3A). Intraperitoneal mCRP injection significantly decreased by 73% the brain expression of PDGFRβ in ApoE2 mice ($p = 0.0058$) but did not influence PDGFRβ expression in mice of the other ApoE genotypes.

Since synapse loss is the key element in cognitive decline in AD (DeKosky & Scheff, 1990; Terry et al., 1991), we next examined whether intraperitoneal mCRP injection influences the expression of synaptic markers across different ApoE genotypes. The expressions of the presynaptic biomarker SYP (Fig. 3B and Supplement Fig. 3B) and the postsynaptic biomarker PSD95 (Fig. 3C and Supplement Fig. 3C) in the brain were not significantly affected among different ApoE knock-in mice regardless of whether they were i.p. injected with PBS or mCRP. Two synaptic markers were shown the spread pattern of imaging, as the markers were confirmed the same tendency in the three repeated experiments. However, intraperitoneal injection of mCRP significantly decreased the brain expression levels of SYP (40% reduction, $p = 0.0253$) and PSD95 (31% reduction, $p = 0.0217$) in ApoE$^{-/-}$ mice (Fig. 3B and Fig. 3C), suggesting that ApoE protein has a protective effect on synapses during peripheral inflammation.

We also examined the influence of elevated peripheral mCRP levels on the development of AD pathology in the brains of mice with different ApoE genotypes. Compared to PBS treatment, elevation of mCRP levels increased neuroinflammation by 32%, as indicated by CD68 expression ($p = 0.0455$) (Fig. 3D and Supplement Fig. 3D), and astrocyte pathology, as indicated by GFAP expression ($p = 0.0422$) (41% increase, Fig. 3E and Supplement Fig. 3E), and significantly induced tau phosphorylation, as detected with a PHF1 antibody (49% increase, $p = 0.0475$) (Fig. 3F and Supplement Fig. 3F), in the hippocampi of ApoE4 mice only. There were no differences in the expression of these AD pathological markers after intraperitoneal injection of PBS; in contrast, following intraperitoneal injection of mCRP, both ApoE4 and ApoE$^{-/-}$ mice had higher levels of these AD markers in the brain than ApoE2 and ApoE3 mice. Elevation of mCRP levels slightly increased the Aβ42 levels in the brains of ApoE4 mice only (Supplementary Figure 3G). Taken together, the data suggested that similar to the lack of ApoE protein in ApoE$^{-/-}$ mice, ApoE4 protein exerts little or no protection against AD pathogenesis in the brain during chronic low-grade inflammation in the body.

3.4. ApoE genotype-dependent relationships between serum ApoE protein expression and the levels of different brain biomarkers

We found that serum ApoE expression had a positive correlation with PDGFRβ expression ($p = 0.0135$) after intraperitoneal PBS injection; however, this correlation was impeded by intraperitoneal mCRP injection (Fig. 4A). Specifically, among ApoE4 mice injected i.p. with mCRP, there was a negative correlation between serum ApoE expression and PDGFRβ levels ($r = -0.6332$, $p = 0.0151$).

Serum ApoE protein expression was not associated with SYP or PSD95 expression in the brain regardless of whether the mice were injected i.p. with PBS or mCRP (Supplement

Figure 4, Fig. 4B). However, in ApoE4 mice, following intraperitoneal mCRP injection, the serum ApoE4 protein level was negatively correlated with PSD95 expression in the brain (r = −0.5571, $p = 0.0385$) (Fig. 4B), suggesting that the toxic structure of ApoE4 protein and its low concentration played some roles.

Additionally, we observed that the serum ApoE protein concentration had negative correlations with the brain levels of neuroinflammation, as indicated by CD68 (r = −0.3766, $p = 0.0140$) (Fig. 4C), astrocyte pathology, as indicated by GFAP (r = −0.3086, $p = 0.0447$) (Fig. 4D), and tauopathy, as detected by a PHF1 antibody (r = −0.3888, $p = 0.0109$) (Fig. 4E) after intraperitoneal mCRP injection only. We did not find correlations between serum ApoE protein and brain Aβ levels in this experiment (data not shown). Taken together, the data suggest that serum ApoE4 exerts less protection than ApoE2 and ApoE3 against AD pathogenesis in the presence of peripheral chronic low-grade inflammation.

3.5. ApoE genotype-dependent relationships between brain ApoE-LRP1 binding and the levels of different brain biomarkers

First, we observed that after intraperitoneal PBS injection (Fig. 5A), brain ApoE-LRP1 binding was positively correlated with PDGFRβ expression (r = 0.6270, $p < 0.0001$), but this relationship disappeared after intraperitoneal mCRP injection. Furthermore, there was a positive correlation between brain ApoE-LRP1 binding and PDGFRβ expression in ApoE2 mice (r = 0.6498, $p = 0.0162$) but not in ApoE3 and ApoE4 mice after PBS treatment.

Brain ApoE-LRP1 binding was positively correlated with SYP expression after PBS treatment (r = 0.4201, $p = 0.0078$), especially in ApoE2 (r = 0.6255, $p = 0.0167$) and ApoE3 (r = 0.6111, $p = 0.0265$) mice, but this relationship was not observed in ApoE4 mice (Fig. 5B). The positive correlation between brain ApoE-LRP1 binding and the expression of SYP in ApoE2 and ApoE3 mice disappeared after intraperitoneal mCRP injection. ApoE-LRP1 binding in the brain was also positively correlated with PSD95 expression after intraperitoneal PBS injection (r = 0.6988, $p = 0.0115$), especially in ApoE2 and ApoE4 mice (Fig. 5C); however, again, this association disappeared when the mice were i.p. injected with mCRP.

In contrast to serum ApoE protein expression (Fig. 4C–E), the binding of ApoE-LRP1 in the brain was not associated with features of AD pathology, i.e., CD68 levels and tauopathy, regardless of the treatment (Supplement Figure 5A and 5B). We observed that ApoE-LRP1 binding had marginal associations with GFAP expression in the brain after intraperitoneal PBS injection (r = −0.2155, p = 0.1938) and intraperitoneal mCRP injection (r = −0.3655, $p = 0.0173$) (Fig. 5D). We did not find correlations between ApoE-LRP1 binding and brain Aβ levels in this experiment (data not shown).

4. Discussion

This is a translational study that used both human data and ApoE knock-in mouse models. We used the FHS cohort and the ApoE mouse models to confirm the conclusion that ApoE genotypes determine the ApoE protein levels (ApoE2>ApoE3>ApoE4) as well as the dominant negative effect of ApoE4 protein on cognition reported by previous studies

(Mooijaart et al., 2006; Marais, 2019; Johnson et al. 2014; Lane-Donovan and Herz, 2016; Torres et al. 2020). We also found that ApoE4-related AD pathology is related to elevation of peripheral mCRP and blood ApoE protein levels through the binding of ApoE-LRP1 in the brain. Although multiple studies have suggested that neuroinflammation is involved in ApoE4-mediated AD pathogenesis in the brain (Kloske and Wilcock, 2020; Tai et al., 2015; Williams et al., 2020), it is unclear whether and how peripheral ApoE and ApoE-LRP1 binding impact AD pathogenesis during peripheral chronic low-grade inflammation. Our study shows that chronic intraperitoneal injection of mCRP induced multiple pathological features of AD in the brain, especially neuroinflammation and tau phosphorylation (pTau), with a severity pattern of ApoE4≥ApoE$^{-/-}$>ApoE3>ApoE2 (Fig. 3). Our study suggests that ApoE4 had the poorest protective ability and the toxic effects, resulting in a greater AD risk than other ApoE genotypes, as both the ApoE4 protein itself and the low ApoE concentration are affected by mCRP. In contrast, higher levels of ApoE and ApoE-LRP1 binding in ApoE2 carriers were associated with higher expression of biomarkers of the BBB and synapses in the brain, which suggests that the ApoE2 allele is protective against AD (Tanzi, 1999; Tao et al., 2018).

In peripheral chronic inflammatory diseases, the blood levels of CRP and mCRP (Hart et al., 2020; Potempa et al., 2021; Rajab et al., 2020; Wu et al., 2015), which are first exposed to the BBB in the brain, are increased. Serum ApoE protein levels and brain ApoE-LRP1 binding were positively associated with the expression of biomarkers of pericytes in the BBB (Figs. 4A and 5A), suggesting that ApoE, through binding to LRP1, plays a role in maintaining BBB integrity, another key element of AD pathogenesis. When the peripheral mCRP level was elevated, the level of degree ApoE-LRP1 binding was reduced in ApoE4 mice (Fig. 2G), and the positive relationship between the serum ApoE level or degree of ApoE-LRP1 binding in the brain and the expression of PDGFRβ disappeared (Figs. 4A and 5A). Several studies have shown that the ApoE4 allele promotes the impairment of the BBB via pericyte degeneration and leads to reduced BBB integrity (Halliday et al., 2016; Montagne et al., 2020; Salloway et al., 2002; Zipser et al., 2007). Taken together, these findings indicate that peripheral chronic inflammation results in the release of mCRP and probably impedes the binding of ApoE-LRP1, causing BBB damage in ApoE4 carriers and leading to AD pathogenesis.

Many studies have shown that the number of synapses in the brain directly correlates with cognitive decline in AD (Reddy and Beal, 2008; Selkoe and Hardy, 2016; Sheng et al., 2012); however, how ApoE protein influences synapses in the brain is not clear. Our study demonstrated that brain ApoE-LRP1 binding, but not serum ApoE, was linked with the expression of the presynaptic biomarker SYP and the postsynaptic biomarker PSD95 in the intraperitoneal PBS injection group only (Fig. 5). It is possible that ApoE is involved in synapse formation in the brain through binding to LRP1. However, after intraperitoneal mCRP injection, the relationships between ApoE-LRP1 binding and the expression of these two synaptic biomarkers no longer existed, probably resulting in increased vulnerability to cognitive decline in ApoE4 carriers. Previous studies have suggested that LRP1 interacts with synaptic proteins, including PSD95 and NMDA receptors (Maier et al., 2013; May et al., 2004). While our study showed that mCRP significantly reduced SYP and PSD95 expression in ApoE$^{-/-}$ mice only (Fig. 5), deletion of neuronal LRP1 leads to synapse

loss, neuroinflammation, and memory loss by impairing lipid metabolism (Liu et al., 2010). Recent report showed that humanized ApoE4 livers are associated with reduction of synaptic integrity, impairment of insulin signaling, and neuroinflammation in hippocampus (Giannisis et al. 2022). Thus, the binding of ApoE and LRP1 after chronic inflammation, which is sufficient in ApoE2 carriers but decreased in ApoE4 carriers plus the ApoE4 toxic structure, could have a key role in neuronal function, contributing to cognitive resilience in ApoE2 carriers and vulnerability to neurodegeneration in ApoE4 carriers.

Both human (Supplement Table 2) and mouse (Fig. 4) illustrate that because of ApoE4 structure and low concentration, serum ApoE4 protein did not have ability to protect from the formation of AD pathology during peripheral inflammation. The experimental data revealed that ApoE2 carriers had the highest protein concentration of serum ApoE, followed by carriers of ApoE3 and ApoE4 (Fig. 1 and 2). In contrast, the protein levels of blood ApoE further declined during the aging process in ApoE4 carriers only (Fig. 1B). While elevation of mCRP levels induced more significant AD pathology in the ApoE4 mouse brain than in the brains of ApoE3 and ApoE2 mice, peripheral ApoE likely plays different roles in antagonizing mCRP in AD pathogenesis due to both the different structures and concentrations of the ApoE alleles. In addition to its specific protein structure, other human studies have shown that ApoE4 carriers have a low plasma concentration of ApoE, which is associated with high AD risk (Prendecki et al., 2019), and that blood ApoE levels are significantly associated with cortical amyloid pathology observed by PiB imaging (Lazaris et al., 2015).

Although ApoE-LRP1 binding was associated with synapses (Fig. 5B and 5C), unlike the serum ApoE protein level, brain ApoE-LRP1 binding had no or minimal impacts on tauopathy and neuroinflammation in the brain (Fig. 5 and Supplement Figure 5). Multiple studies have shown that LRP1 is a major receptor that binds to the ApoE protein (Holtzman et al., 2012; Kanekiyo and Bu, 2014; Shinohara et al., 2017). Indeed, we used the PLA technique to confirm that the ApoE protein bound to LRP1 in the brain in the pattern of ApoE2>ApoE3>ApoE4 and found that elevation of peripheral mCRP levels further reduced ApoE-LRP1 binding in the brain (Fig. 2G). While LRP1 plays an important role in AD pathogenesis through Aβ pathways (Holtzman et al., 2012; Kanekiyo and Bu, 2014; Shinohara et al., 2017), we did not find that ApoE-LRP1 binding was associated with neuroinflammation and tauopathy in this experiment (Fig. 5). Most likely, during peripheral chronic inflammation, ApoE binds to different receptor(s) other than LRP1 to protect against the development of the typical pathological features of AD, especially tauopathy. According to the literature, other receptors binding the ApoE protein include VLDLR (very low-density lipoprotein receptor) (Christie et al., 1996), ApoER2 (apolipoprotein E receptor 2) (Fan et al., 2001), and LDLR (low-density lipoprotein receptor) (Swanson et al., 1988), which are expressed in neurons, astrocytes, and microglia as well as endothelial cells. Our recent study found that the ApoE protein can also bind CD31, an endothelial membrane protein that antagonizes neuroinflammation and tauopathy in the brain during peripheral chronic low-grade inflammation (Zhang et al., 2021).

Overall, in addition to the distinct protein structures of the different ApoE alleles, the high serum ApoE level in ApoE2 carriers and low ApoE level in ApoE4 carriers are also a

determining factor for AD risk in the presence of chronic low-grade inflammation during the aging process (Figure 6). High protein levels of blood ApoE could lead to increased ApoE-LRP1 binding in the brain and contribute to BBB integrity and synaptic function, especially in ApoE2 carriers, which might enhance resilience to the inflammation that occurs during the aging process.

5. Conclusion

Our study demonstrates that the protein levels of blood ApoE and ApoE-LRP1 binding in the brain were determined by ApoE genotype in the order ApoE2>ApoE3>ApoE4. Both the blood ApoE protein concentration, ApoE isoform structure and degree of ApoE-LRP1 binding in the brain had positive correlations with the number of pericytes in the BBB and synapses in the brain; elevation of peripheral mCRP levels impaired these relationships, especially in ApoE4 carriers. Because the blood-facing part of the BBB is exposed to blood mCRP and ApoE during peripheral inflammation, our study suggests that peripheral mCRP produced during chronic inflammation and the ApoE protein have competitively opposite actions on the development of cerebrovascular and AD pathologies in the brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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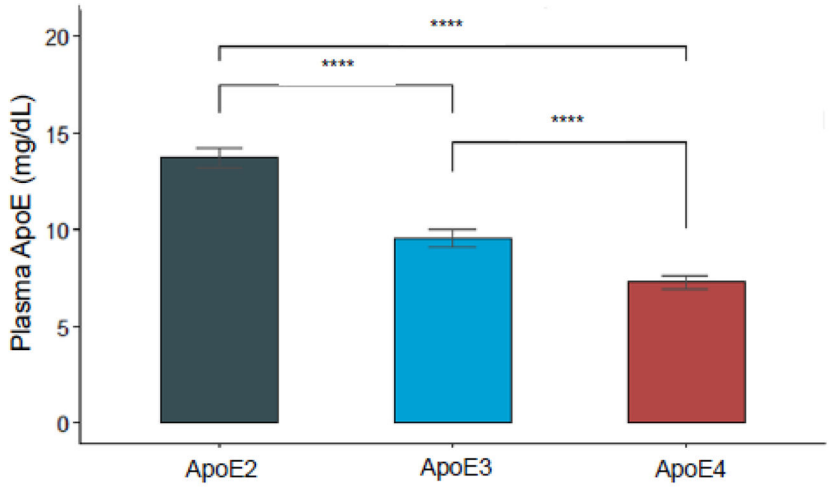
Highlights

- The older the ApoE4 carriers, the lower level of blood ApoE protein.
- Blood ApoE protein and brain ApoE-LRP1 binding are protective for AD risk.
- Blood ApoE protein is positively associated with pericytes.
- Brain ApoE-LRP1 binding is positively associated with synaptic markers.
- ApoE is protective in the pattern of ApoE2>ApoE3>ApoE4 during inflammation.

Fig. 1A



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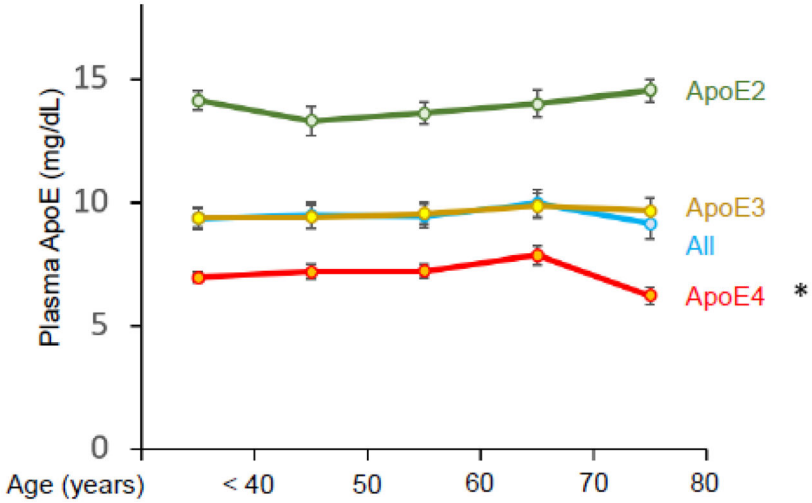


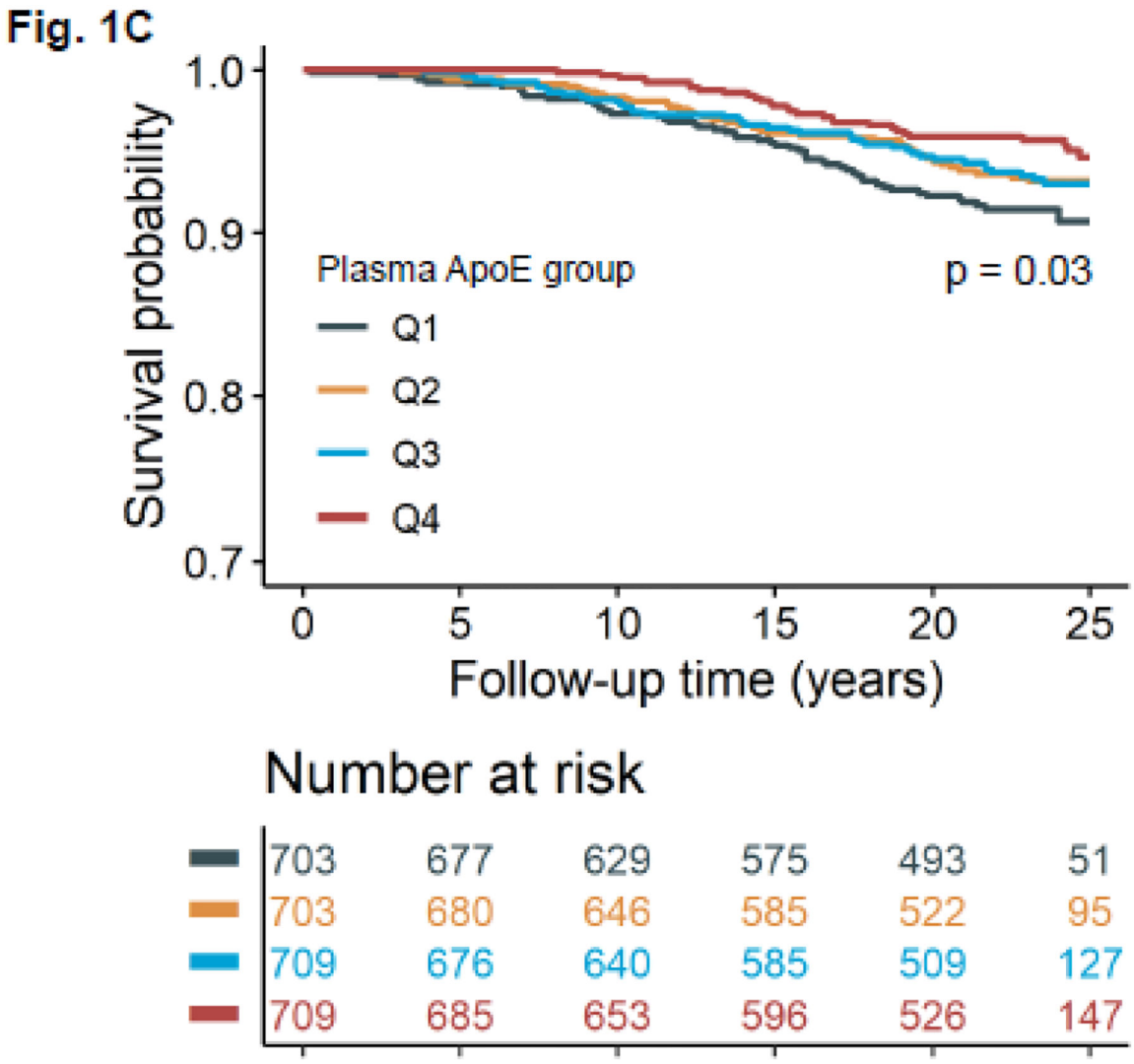
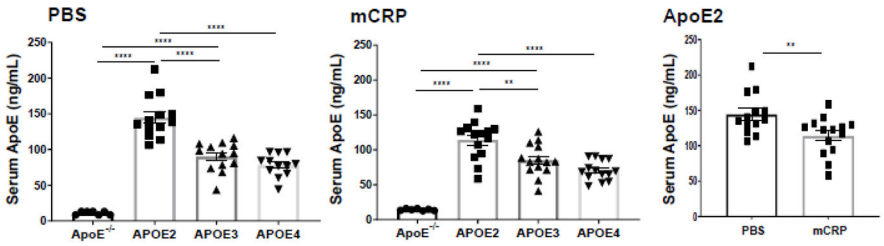


Fig. 1. Plasma ApoE protein levels and Alzheimer's disease risk in the FHS study

FHS dataset was used to examine the relationship between plasma ApoE protein and AD risk across ApoE genotypes in humans. (**A**) Plasma ApoE protein levels associated with ApoE genotypes showing that, on average, ApoE2 carriers had the highest protein concentration of plasma ApoE followed by ApoE3 carriers and then ApoE4 carriers (mean ± SD [mg/dL]: 14.2 ± 5.4 vs. 10.1 ± 4.4 vs. 7.6 ± 3.3, p <0.0001). (**B**) Association between plasma ApoE protein levels and aging showing that, only in ApoE4 carriers, the average plasma ApoE concentration significantly decreased as age increased. (**C**) Kaplan–Meier (KM) plot showing that a lower level of plasma protein increased the risk of AD. Subjects were divided into four quartiles (Q1, Q2, Q3 and Q4) according to plasma ApoE concentration. Those in Q4, who had the highest protein concentration of plasma ApoE, had a lower risk of AD, followed by those in Q3 and Q2; those with the lowest protein concentration of plasma ApoE (Q1) had a higher risk of AD (log-rank test, p = 0.03).

Fig. 2A



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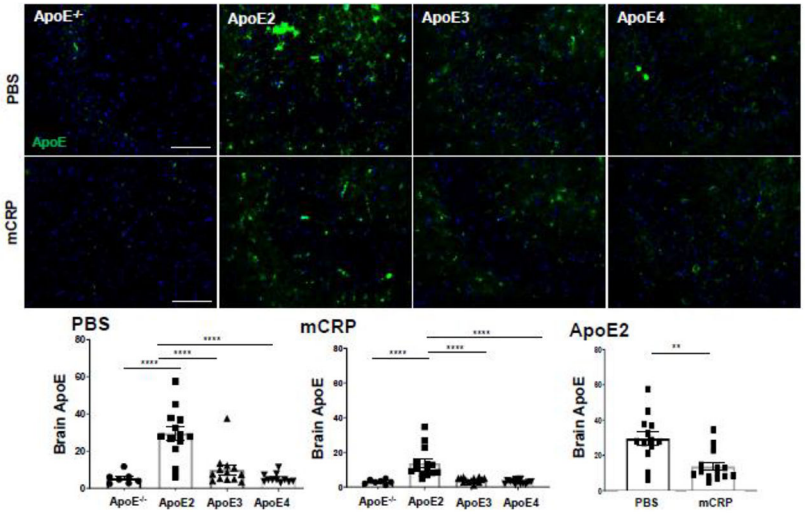

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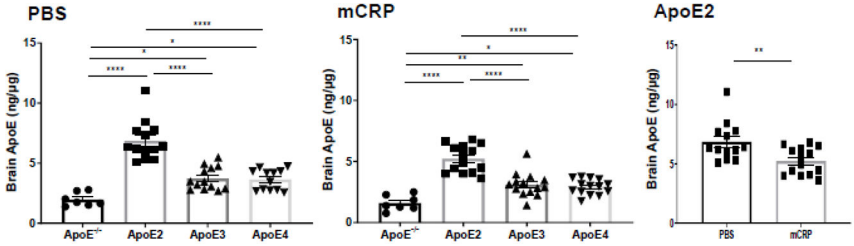

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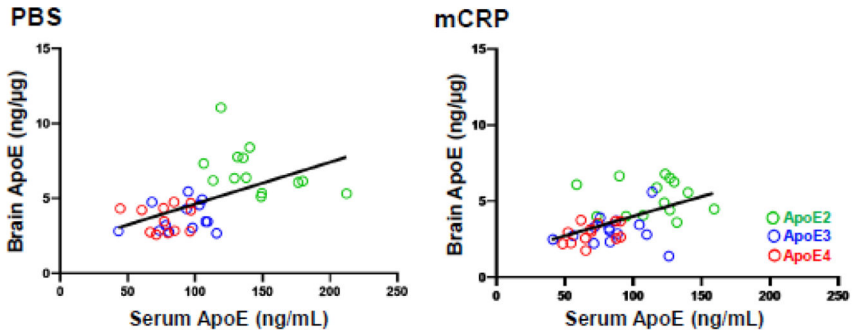

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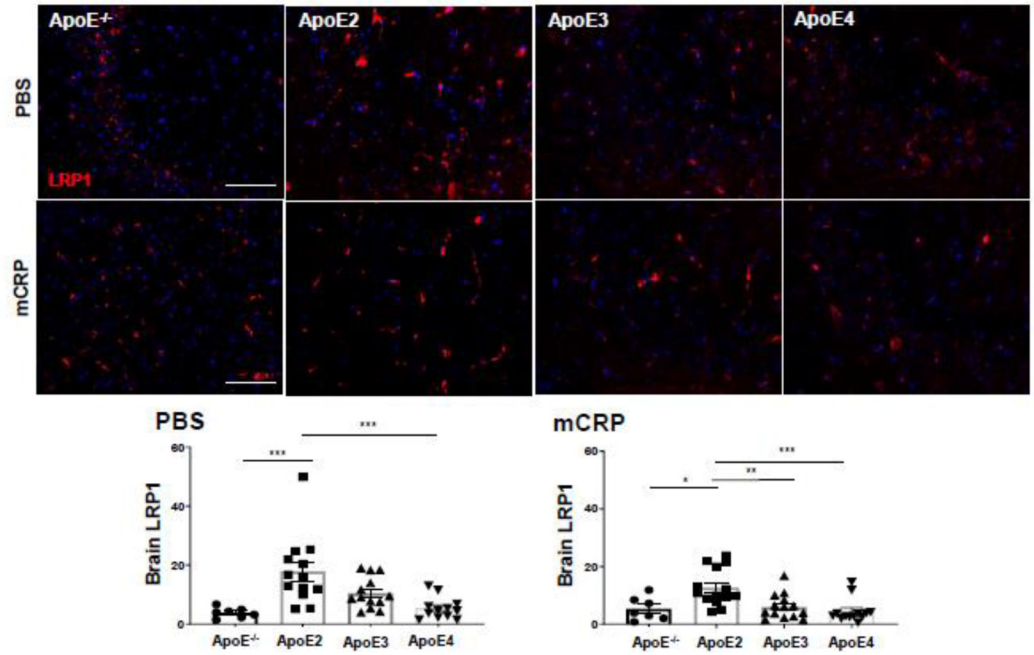

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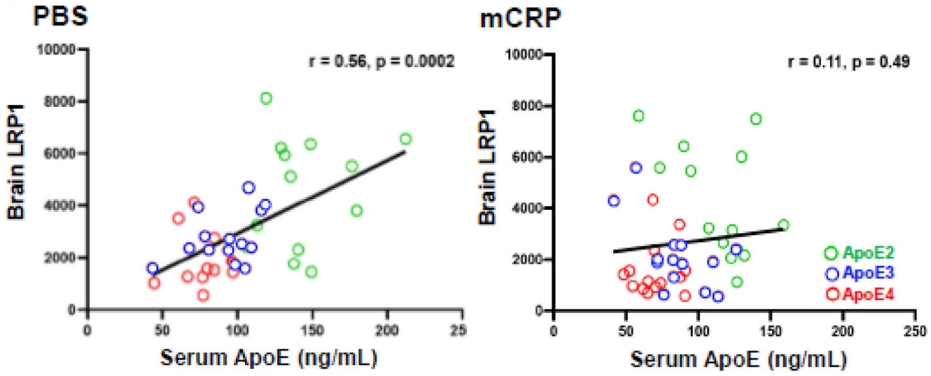


Fig. 2G

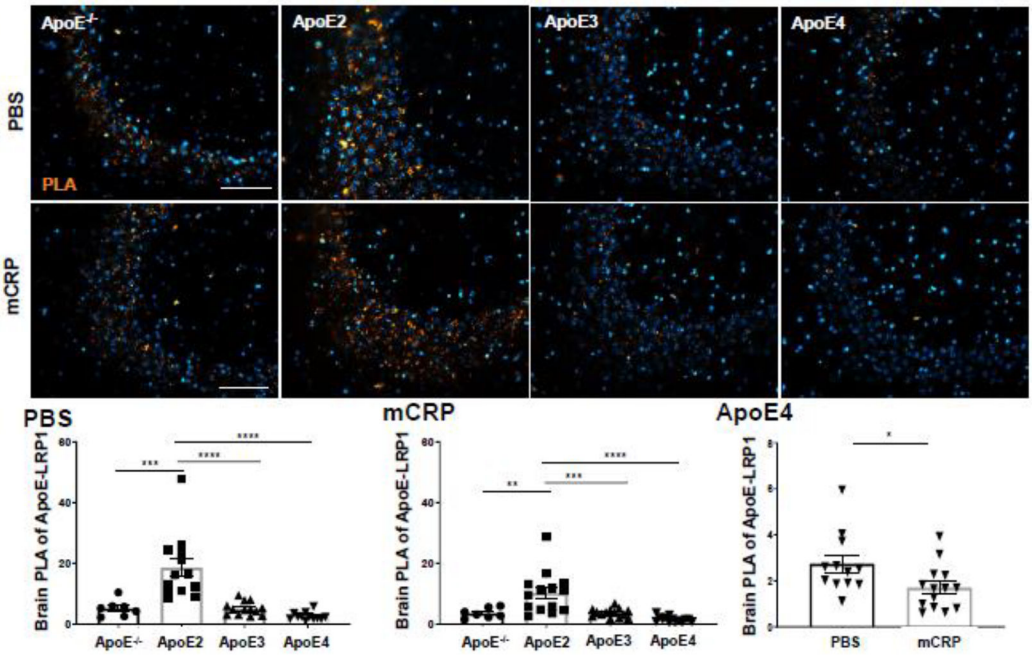


Fig. 2H

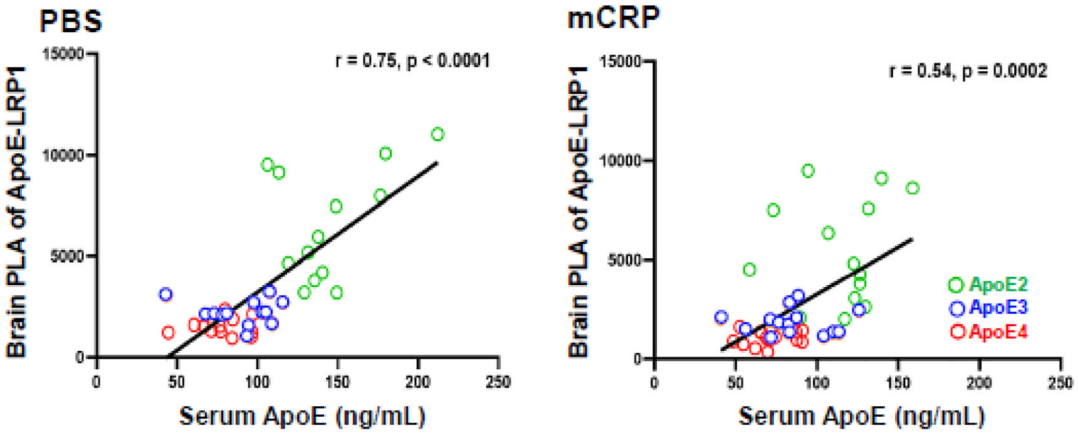


Fig. 2. The peripheral inflammatory factor mCRP reduces ApoE and LRP1 levels

Ten-month-old female ApoE$^{-/-}$, ApoE2, ApoE3, and ApoE4 mice were used and were intermittently injected i.p. with PBS or mCRP for 6 weeks and were compared either across different ApoE genotypes in the PBS and mCRP groups or between two i.p. treatments. (**A**) Blood serum levels of ApoE were measured by ELISA (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). (**B**) ApoE expression in sections of the hippocampal CA3 region was assessed by immunohistochemistry (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, scale bar: 200 μm). (**C**) ApoE expression in the hippocampus was examined by ELISA (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). (**D**) Correlations between ApoE levels in the serum and brain were analyzed in mice of different ApoE genotypes in the PBS and mCRP groups. (**E**) Brain sections were stained with an anti-LRP1 antibody (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, scale bar: 200 μm). (**F**) Correlations between the serum levels of ApoE and LRP1 were analyzed. (**G**) Proximity ligation assay (PLA) of the interaction of ApoE with LRP1. Orange signals indicate binding between ApoE and LRP1 (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, scale bar: 200 μm). (**H**) Positive correlations between the degree of ApoE-LRP1 binding and serum ApoE levels were observed. Third panels in Fig 2A–C and 2G are emphasized figure from all groups. The images were calculated the percentage by normalization with DAPI. Each group is indicated by a different shape (ApoE$^{-/-}$, circle; ApoE2, square; ApoE3, triangle; ApoE4, inverted triangle). Correlation data for each group are presented in a different color (ApoE2, green; ApoE3, orange; ApoE4, red). The data are presented as the mean ± SE. For all comparisons, statistical analyses (ANOVA followed by Tukey's test and Student's *t* test) were performed. Pearson's correlation was used to assess correlations between serum and brain protein levels.

Fig. 3A

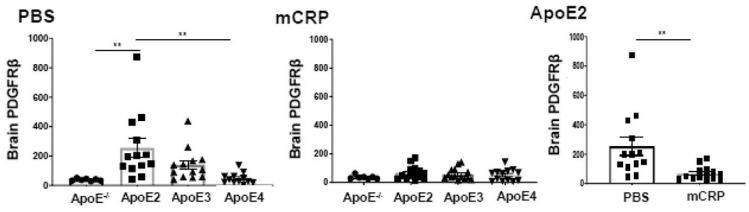


Fig. 3B

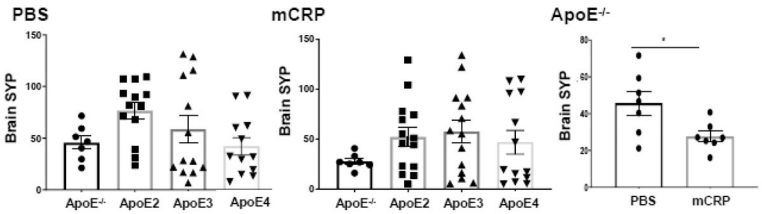


Fig. 3C

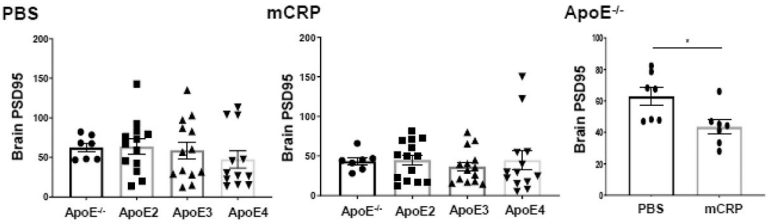
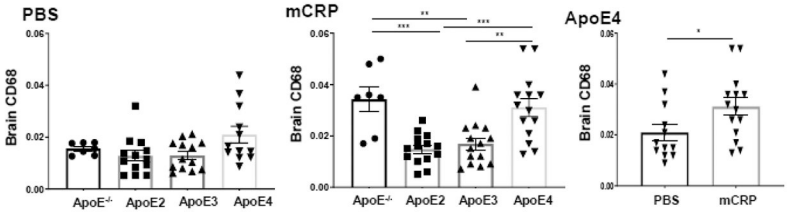


Fig. 3D



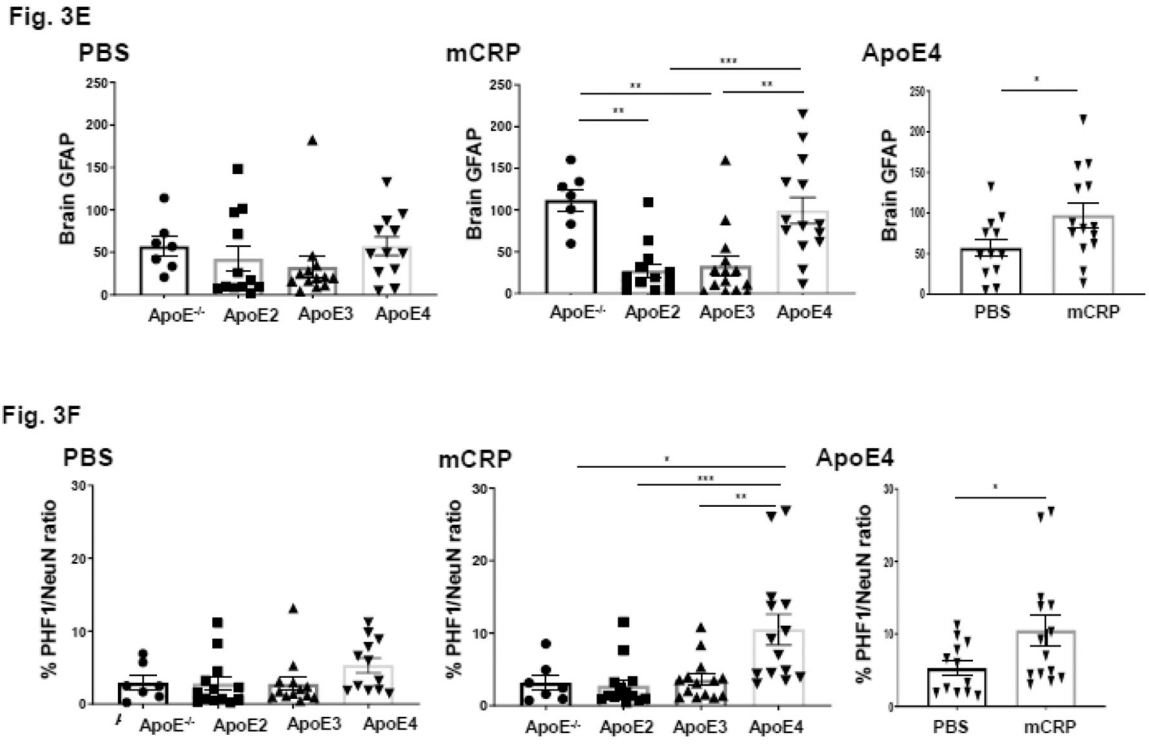


Fig. 3. The peripheral inflammatory factor mCRP reduces the expression of a pericyte marker and induces AD pathology across ApoE genotypes

Brain proteins and AD pathological markers were detected in brain sections from mice of different ApoE genotypes that were i.p. injected with PBS vs. mCRP. Different genotypes were compared under i.p injection of either PBS or mCRP; Two i.p. treatments were compared in each ApoE genotype. (**A**) PDGFRβ expression in sections of the hippocampal CA3 region was examined by immunostaining (**$p < 0.01$). (**B**) The brain sections were stained with an anti-synaptophysin (SYP) antibody (*$p < 0.05$). (**C**) The brain sections were stained with an antibody against a postsynaptic marker (PSD95) (*$p < 0.05$). **(D)** CD68 expression in sections of the hippocampus was examined by immunostaining (*$p < 0.05$, **$p < 0.01$). (**E**) GFAP expression in astrocytes (*$p < 0.05$, **$p < 0.01$). **(F)** Tau phosphorylation (pTau) was assessed by staining brain sections with an anti-PHF1 antibody (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). PHF1 expression was normalized by NeuN expression. In addition, mCRP treatment significantly increased the expressions of CD68, GFAP and PHF1 pTau in ApoE4 mice compared to mice of another genotypes (*$p < 0.05$). Third panels in Fig 3 are emphasized figure from all groups. The images were calculated the percentage by normalization with DAPI. The data are presented as the mean ± SE. For all comparisons, statistical analyses (ANOVA followed by Tukey's test and Student's *t* test) were performed.

Fig. 4A

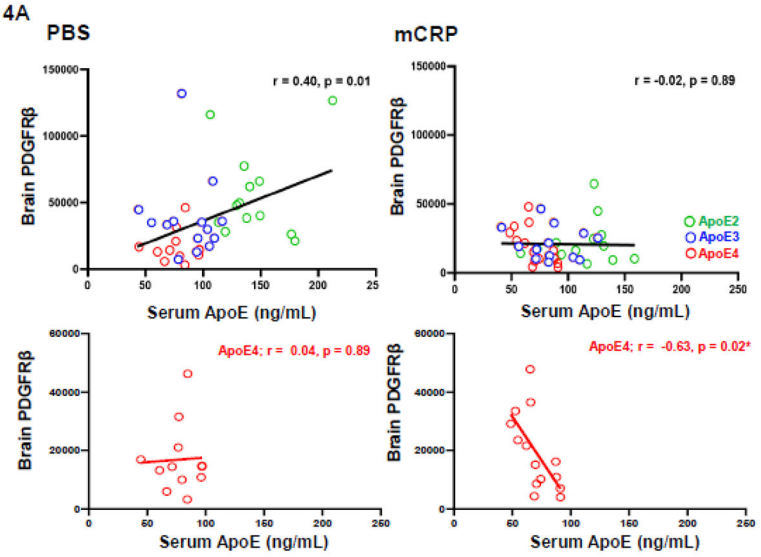
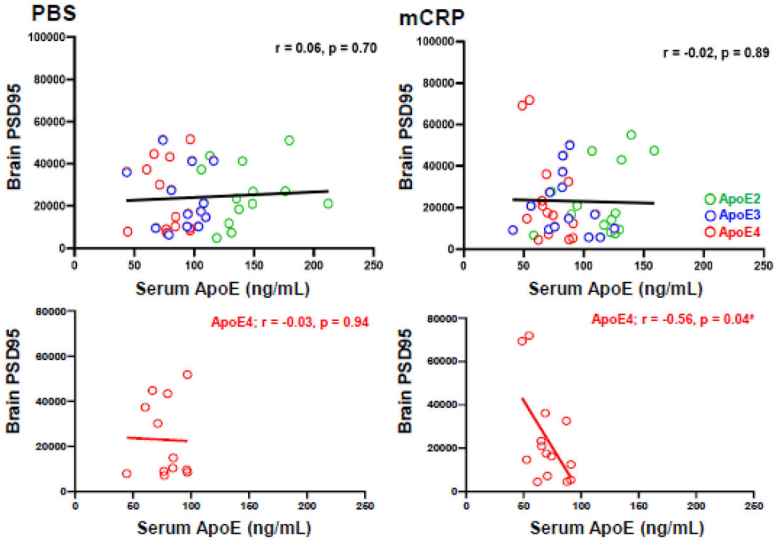


Fig. 4B



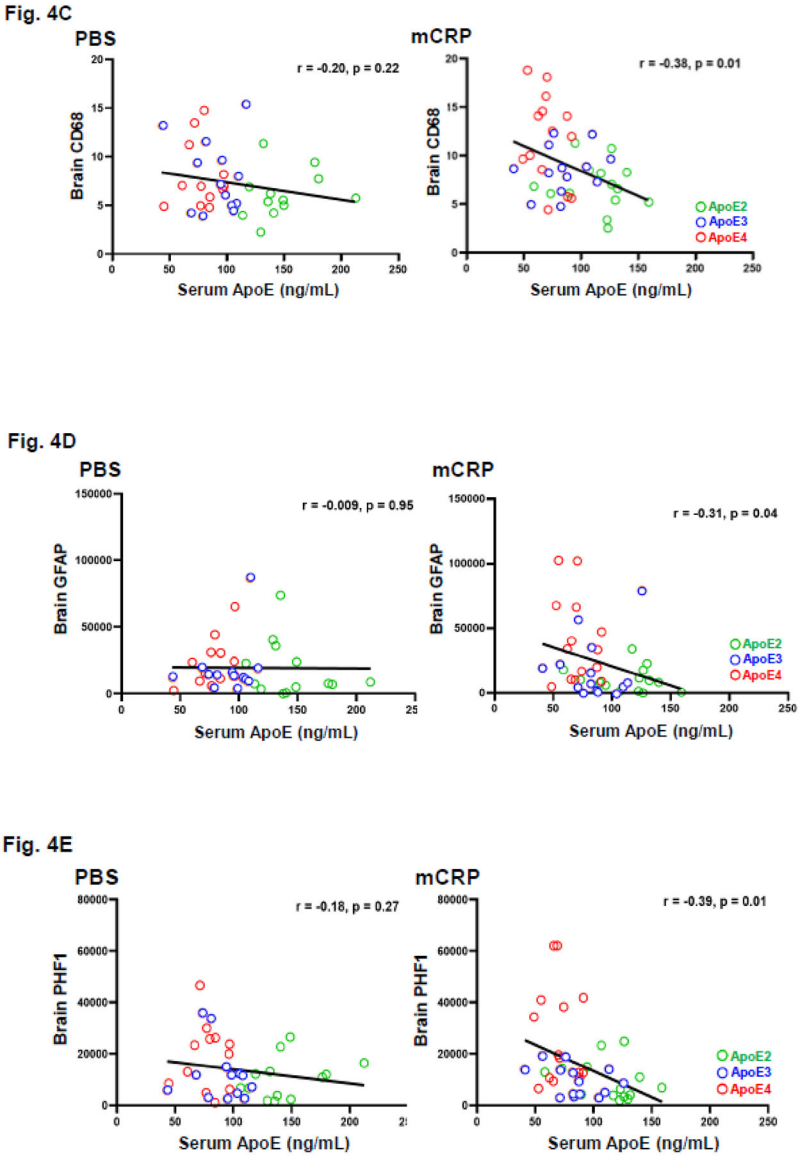


Fig. 4. The peripheral inflammatory factor mCRP impedes the positive relationships among blood ApoE protein expression, the number of pericytes, and AD pathology

Pearson's correlation was used to analyze the correlations among the serum ApoE level, PDGFRβ expression, synaptic proteins and AD pathology. (**A**) Correlations between serum ApoE levels and PDGFRβ expression were analyzed in mice of different ApoE genotypes that were treated with PBS or mCRP. (**B**) The correlation between the serum ApoE level and brain PSD95 expression is shown. (**C**) Correlations between CD68 expression and the serum ApoE level were analyzed. (**D**) Correlations between GFAP expression and serum ApoE levels were assessed. (**E**) Correlations between PHF1 staining and the serum ApoE level in the PBS and mCRP groups were evaluated. Correlation data for each group are presented in a different color (ApoE2, green; ApoE3, orange; ApoE4, red). If the correlation data was significant in any ApoE genotype, the correlation for the ApoE genotype was shown in a separate graph. P values are shown.

Fig. 5A

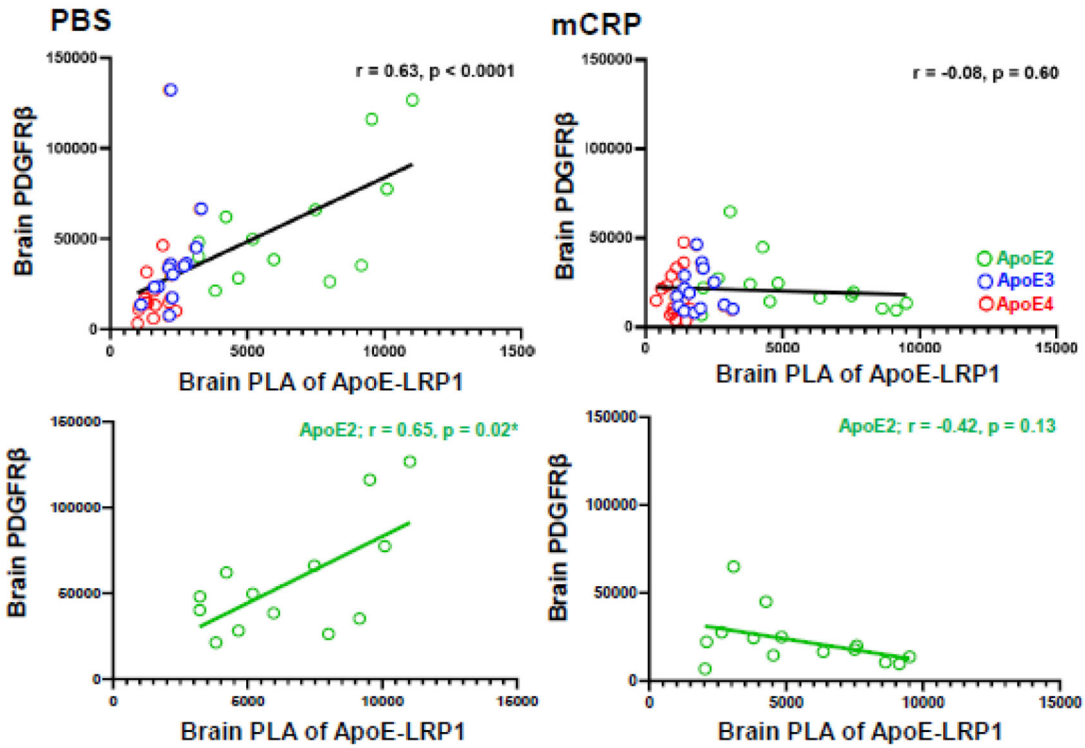


Fig. 5B

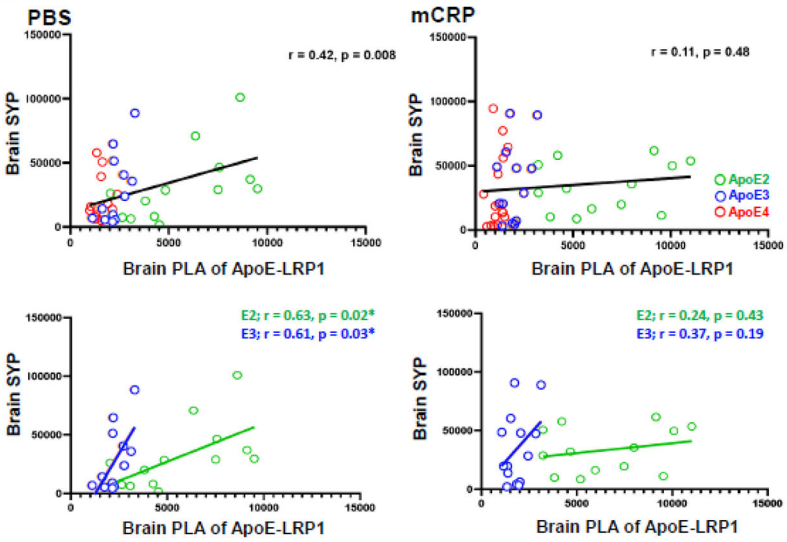


Fig. 5C

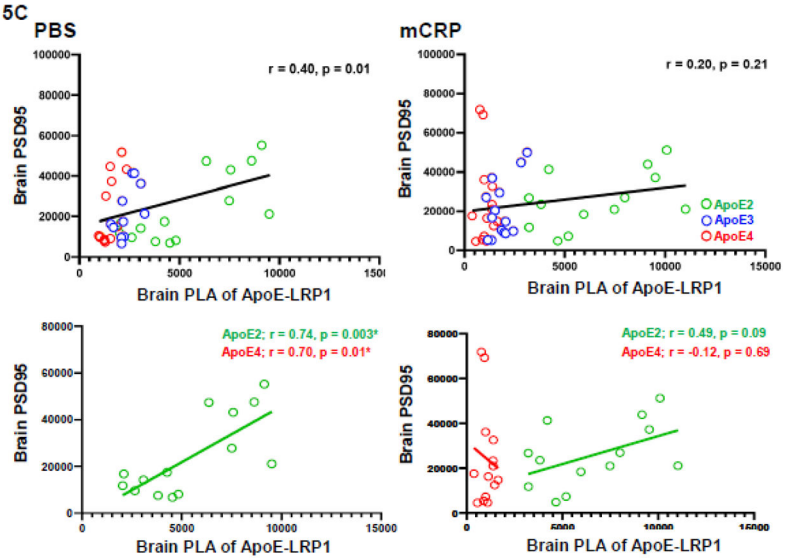


Fig. 5D

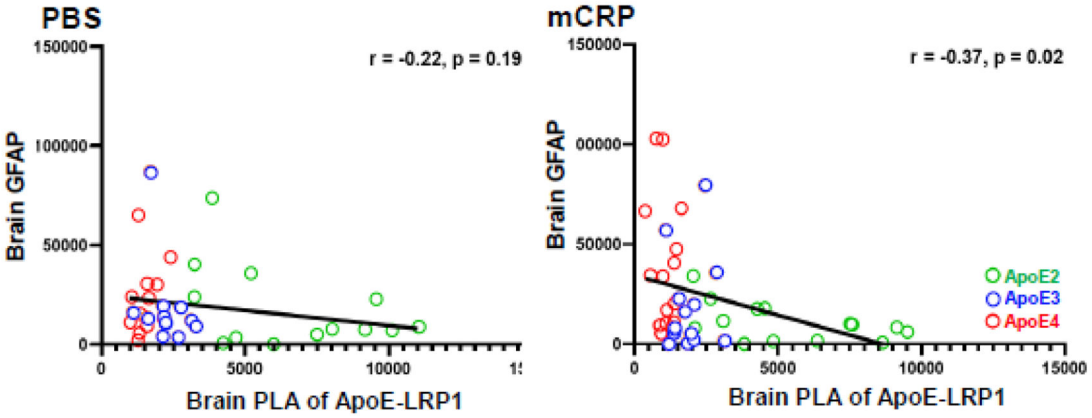


Fig. 5. The peripheral inflammatory factor mCRP impedes the positive relationships among the degree of brain ApoE-LRP1 binding, the number of pericytes and AD pathology

Pearson's correlation was used to analyze the correlations among the brain ApoE-LRP1 level, PDGFRβ expression, synaptic proteins and AD pathology. **(A)** Correlations between brain PLA of ApoE and LRP1 and PDGFRβ expression were analyzed in mice of different ApoE genotypes that were treated with PBS or mCRP. (**B**) Correlations between PLA of ApoE-LRP1 binding and SYP expression in the brain are shown for each ApoE genotype. **(C)** Correlations between PLA of ApoE-LRP1 binding and PSD95 expression in the brain are shown for each ApoE genotype. **(D)** Correlations between GFAP expression and PLA of ApoE-LRP1 binding in the brain were calculated. Correlation data for each group are presented in a different color (ApoE2, green; ApoE3, orange; ApoE4, red). If the correlation data was significant in any ApoE genotype, the correlation for the ApoE genotype was shown in a separate graph. P values are shown.

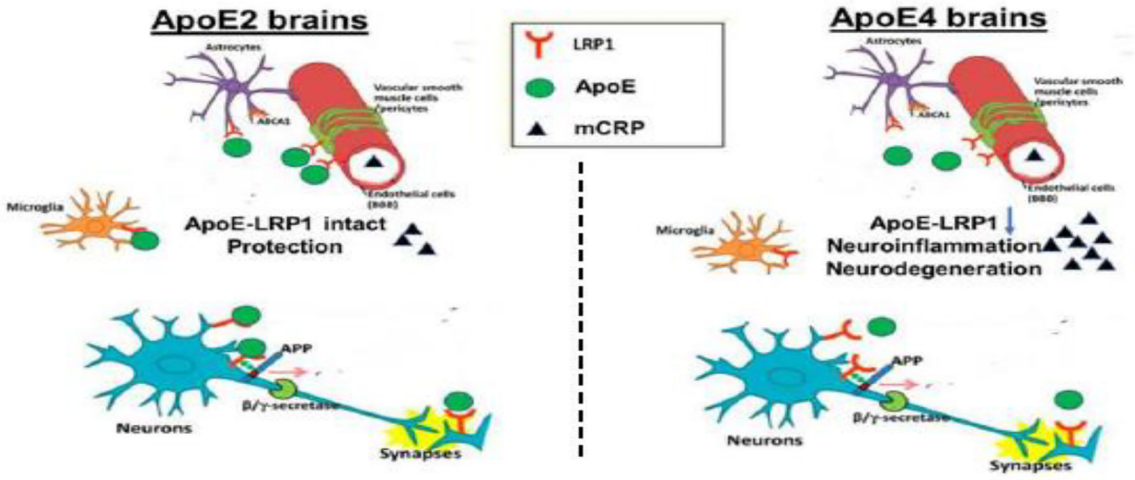


Fig. 6. A model illustrating the differential responses of ApoE2 vs. ApoE4 carriers to mCRP on the ApoE-LRP1 binding leading to different alterations in the brain.

This study suggested that the binding of ApoE protein and LRP1 receptor plays an important role in neural protection during peripheral low-grade inflammation which releases mCRP. mCRP disturbed the binding of ApoE4, but not ApoE2, protein and LRP1 receptor in different neural cells in the brain. Because of ApoE4 protein structure and low concentration for less ApoE4-LRP1 binding, ApoE4 brain was vulnerable to respond to mCRP leading to the BBB damage, neuroinflammation, astrocyte pathology, neurodegeneration and synapsis loss. Because of ApoE2 structure and high concentration to have more ApoE2-LRP1 binding, ApoE2 brain was protected to respond to mCRP.