Supporting Information

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SI Materials and Methods

Reagents. Amyloid-β (Aβ)(1-40) and Aβ(1-42) were purchased from American Peptide Co. ^{3}H-Cholesterol was purchased from Perkin-Elmer Life Sciences (Waltham, MA). Recombinant human apolipoprotein E (apoE) isoforms were purchased from Leinco Technologies (St. Louis). Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL).

Animals. Homozygous PDAPP (APPV717F) mice lacking apoE on a mixed background comprised of DBA/2J, C57BL/6J, and Swiss Webster were crossed with mice expressing the apolipoprotein E gene *APO ε2*, *ε3*, and *ε4* under control of mouse regulatory elements on a C57BL/6J background (a gift from P. Sullivan, Duke University, Winston-Salem, NC) (1). Mice then were intercrossed to create homozygous PDAPP/targeted replacement of apoE (TRE) mice (a gift from Ronald B. DeMattos, Eli Lilly and Co., Indianapolis). Age- and sex-matched PDAPP/TRE mice were used throughout experiments. All animal procedures were performed according to protocols accepted by the Animal Studies Committee at Washington University School of Medicine.

Preparation and Characterization of Reconstituted apoE and Astrocyte-Secreted apoE Isoforms. Reconstituted apoE2 (rapoE2), apoE3 (rapoE3), and apoE4 (rapoE4) particles were prepared by a cholate dialysis method using apoE:POPC:cholesterol molar ratios of 1:50:10 and 1:5:1, respectively (2, 3). Samples of the reconstituted apoE (rapoE) particles were analyzed by gel filtration-FPLC and nondenaturing gradient PAGE. Equal amounts of apoE isoforms (6 μg) were loaded in each lane of 4–20% Tris·glycine gel for native–PAGE (100 V at 4 °C for 16 h). The migration pattern of lipoproteins was assessed using a protein mixture of estimated hydrodynamic radii as a standard (GE/Amersham). ApoE-containing lipoprotein particles from mouse primary astrocytes and astrocytes stably expressing human apoE isoforms were collected and prepared by immunoaffinity chromatography, as described previously (4, 5).

Aβ Preparation: H4 Neuroglioma Cells, Chinese Hamster Ovarian Cell Line (7PA2), and Synthetic Aβ. H4 neuroglioma (H4APP695ΔNL) cells were grown in Opti-MEM containing 10% FBS, hygromycin (200 μg/mL), blasticidin (10 μg/mL), and penicillin and streptomycin. At 90% confluence, the cells were washed three times with Opti-MEM or DMEM without FBS and then were incubated with serum-free growth medium. Human Aβ peptides secreted by H4APP695ΔNL cells were collected at 24 h and concentrated to 5× by a 3-kDa cutoff concentrator (Millipore, Amicon Ultra). The Aβ from the concentrated medium was isolated by size exclusion-FPLC (Superdex 75, 10/300 GL, buffer: 50 mM ammonium acetate, pH 8.5, in Milli-Q water). Western blot analysis was used to determine the purity of Aβ isolated from conditioned media (CM). All detectable levels of Aβ were found migrating below the 6-kDa molecular weight marker bands. Aliquots of purified Aβ samples were stored at −80 °C. Aβ from 7PA2 CM medium was purified, as described previously (6). Briefly, cell-secreted human Aβ peptides were collected and concentrated 10-fold by a 3-kDa cutoff concentrator. Aβ from the concentrated medium was isolated by size exclusion-FPLC (Superdex 75, 10/300 GL, buffer: 50 mM ammonium acetate, pH 8.5, in Milli-Q water). Aliquots of purified Aβ samples were stored at −80 °C.

Synthetic Aβ was prepared by reconstituting the lyophilized Aβ(1-40) and Aβ(1-42) peptides in trifluoroacetic acid and incubating the peptides at room temperature for 15 min. The peptides then were dried under nitrogen gas and resuspended in hexafluoroisopropanol (HFIP). The HFIP then was dried under nitrogen gas, yielding a dry Aβ film, which was stored at −80 °C. Before use, the Aβ film was dissolved in DMSO.

Isolation of the ApoE/Aβ Complex by KBr Density-Gradient Ultracentrifugation. To measure apoE and Aβ association in the presence of neuroglioma cells, H4 APP695ΔNL cells were grown to 90% confluence in 9-cm^2 wells (six-well plates) and were washed three times with Opti-MEM or DMEM without FBS. H4 APP695ΔNL cells in serum-free medium then were incubated with lipidated rapoE particles or primary astrocyte-derived mouse apoE particles for 3, 6, or 12 h. The Aβ concentrations present in the medium at 3, 6, and 12 h were ∼50 ng/mL, 80 ng/mL, and 120 ng/mL, respectively (slope = 10 ± 1.5 ng·mL^{-1}·h^{-1}). Samples of medium containing apoE and Aβ were collected at the end of experiments and centrifuged at 150 rcf for 5 min. Samples also were placed at the bottom of the centrifuge tube with a final KBr density (D) of 1.26 g/cm^3 [refractive index (RI) = 1.3750; vol = 2.1 mL] and subsequently were layered with a KBr solution (D = 1.12 g/cm^3; RI = 1.3550; vol = 1.5 mL) and 150 mM NaCl, pH 7.3 (D = 1.0 g/cm^3; RI = 1.3330; vol = 1.2 mL). The samples were centrifuged using a Vti 65.2 rotor (Beckman Coulter) for 105 min with 327,802 × *g* at 8 °C. The density distribution of lipidated apoE in the gradient was determined by apoE ELISA as described below and by ^{3}H-cholesterol radioactivity. Using a sensitive Aβ ELISA as described below, the separated samples were analyzed to determine the concentration of Aβ present in the gradient. The majority of apoE was present at density below 1.15 g/cm^3, and Aβ present in the region corresponding to densities from 1.10–1.15 g/cm^3 was considered to be associated with apoE. To measure apoE isoforms and extracellular Aβ binding, 100 ng/mL of H4 APP695ΔNL Aβ purified from CM by size-exclusion chromatography (SEC) was incubated with 20 μg of lipidated apoE isoforms for 12 h (molar ratio of Aβ:apoE, 1:25). Samples were collected and subjected to KBr gradient centrifugation, and apoE and Aβ levels were determined by ELISA as described above.

Measurement of Aβ and apoE Levels by Sandwich ELISA. Levels of Aβ were measured using sandwich ELISAs; m266 (anti–Aβ13–28) was used as capture antibody, followed by detection with biotinylated 3D6 (anti–Aβ1–5). ApoE levels were quantified using an in-house apoE sandwich ELISA (HJ6.2 for capture and HJ6.1B for detection) with recombinant human apoE as a standard.

SEC. SEC by FPLC was performed with single or tandem Superose-6, 10/300 GL columns (GE Healthcare). Pooled human CSF or CSF from ApoE3/3 and ApoE4/4 human subjects [Clinical Dementia Rating (CDR) = 0] (0.5 mL or concentrated) was eluted with PBS containing 50 mM sodium phosphate, 150 mM NaCl, pH 7.4, and 0.02% EDTA at a flow rate of 0.8 mL/min. The fractions were collected, and the apoE and Aβ levels were determined by ELISA as described above. To measure the association of apoE and Aβ in solution, CSF apoE and exogenous Aβ (50 ng/mL of H4 APP695ΔNL Aβ purified from CM by SEC) were incubated with 800 uL pooled CSF for 6 h. Fractions were collected, and the apoE and Aβ concentrations were determined by ELISA as described above. rapoE3 and apoE4 (20 μg) and astrocyte-secreted and immunopurified apoE3 and apoE4 (10 μg) were incubated in serum-free DMEM-F12 medium containing 100 ng/mL H4 APP695ΔNL purified Aβ for 3 or 6 h. A portion of the samples was loaded on size-exclusion columns and was eluted

with PBS containing 50 mM sodium phosphate, 150 mM NaCl, pH 7.4, and 0.02% EDTA at a flow rate of 0.8 mL/min. The fractions were collected, and the apoE and Aβ were determined by ELISA as described above.

Fluorescence Correlation Spectroscopy. Fluorescence correlation spectroscopy (FCS) measurements were performed on a Confocal 2 microscope equipped with FCS capability (Zeiss Inc.). The FCS autocorrelation data were analyzed to obtain the diffusion time (7–9). TMR-labeled Aβ40 and Aβ42 were prepared as described previously (10). ApoE and Aβ complex formation experiments were performed in a buffer containing (in mM) 20 Hepes, 150 NaCl, and 5 β-mercaptoethanol (β-Me), pH 7.4. To measure the time dependence of diffusion coefficient, 100 nM TMR labeled-Aβ40 and/or Aβ42 was incubated with various concentrations of rapoE and astrocyte-secreted apoE. The samples were incubated in glass coverslip-bottomed eight-well chambers (Nunc) at room temperature. FCS measurements were performed at 2 h and 24 h to follow the time dependence of apoE/Aβ complex formation.

Cholesterol Efflux. Cholesterol efflux efficiency of rapoE acceptors and the effect of Aβ on apoE cholesterol efflux were determined in H4 neuroglioma cells as previously described (11). Briefly, cells were radiolabeled with 1,2-[^{3}H]-cholesterol (2 μCi/mL) in DMEM containing 2.5% FBS. The medium was removed, and the cells were incubated for 1 h in serum-free DMEM containing 0.05% BSA (DMEM/BSA). Before the start of the experiment, the cells were rinsed twice with DMEM/BSA. The labeled cells were incubated for 6 h in the absence or presence of 20 μg/mL of rapoE isoforms. Cholesterol efflux efficiency of apoE in the presence of Aβ was determined; radiolabeled H4 cells were incubated with apoE3 (20 μg) and 0 nM, 3.75 nM, 7.5 nM, or 15 nM CM containing H4 APP695ΔNL cell-derived Aβ. Aliquots of the medium were collected at specific time intervals, and the 1,2-[^{3}H]-cholesterol was determined by scintillation counting. At the end of the incubation, the cells were washed twice with PBS, and the lipids were extracted with isopropanol. The lipid extract was used to determine the fraction of radiolabeled cholesterol remaining in the cells. Cholesterol efflux was expressed as the percentage of radiolabeled cholesterol released into the medium as 100 *[^{3}H]-Cholesterol in medium/([^{3}H]-Cholesterol in medium + [^{3}H]-Cholesterol in cells).

In Vivo Microdialysis. In vivo microdialysis in 3- to 4.5-mo-old PDAPP/TRE mice was performed essentially as described to assess the steady-state concentration of Aβ present in the hippocampal interstitial fluid (ISF), this time using a 38-kDa cutoff dialysis probe that allowed the infusion of large macromolecules directly at the site of dialysis using a side infusion port (Bioanalytical Systems, Inc.) (12, 13). Soluble, exchangeable ISF $Aβ_{1–x}$ ($eAβ_{1–x}$) was collected using a flow rate of 1.0 μL/min before and after acute infusion of 1 μg of rapoE2 or rapoE4 particles (in artificial CSF). Infusion of rapoE particles was performed after a baseline period of sampling at a flow rate of 0.07 μL/min using a calibrated nano-injector infusion pump (Stoelting). Quantitative measurements of eAβ collected from microdialysis fractions were made using sensitive sandwich ELISAs as described above.

Immunoblots. Human astrocytes expressing apoE isoforms apoE2, apoE3, and apoE4 and apoE-KO immortalized astrocytes were lysed in RIPA buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 25 mM Tris·HCl, 150 mM NaCl) to measure binding immunoglobulin protein (BiP), C/EBP homology protein (CHOP), inositol-requiring enzyme 1 α (IRE1α), programmed death-1 (PD-1), and tubulin. The lysates were spun down at 14,000 × *g* for 20 min, and the supernatant was collected. Protein concentration was determined by a bicinchoninic acid (BCA) protein assay. Equal amounts of protein for each sample were run on 4–12% Bis-Tris gels and were transferred to nitrocellulose membranes (0.2-μM pore size). All membranes then were blocked in 5% milk in Tris-buffered saline with 0.125% Tween 20. Blots were probed for the endoplasmic reticulum (ER) stress markers BIP, CHOP, IRE1-α, and PDI (Cell Signaling Technology, ER Stress sampler kit, catalog number 9956; antibody dilution, 1:1,000). The protein signal from the membranes was measured using a Lumigen TMA-6 ECL detection kit (Lumigen) and quantified using ImageJ software (National Institutes of Health).

Surface Plasmon Resonance Spectroscopy. Sensor chips were purchased from GE-BIAcore. All surface plasmon resonance (SPR) experiments were carried out on a BIAcore 2000 instrument at 25 °C. Lyophilized Aβ(1-40) and Aβ(1-42) peptides were prepared as described in *SI Materials and Methods*. Aβ(1-40) and Aβ(1-42) were immobilized onto a CM5 sensor chip surface at densities of ∼4–5 fmol/mm^2 by amine coupling with sodium citrate buffer, pH 4.75, according to the manufacturer's instructions (BIAcore AB). One flow cell was activated and blocked with 1 M ethanolamine without any protein and was used as a control surface to normalize SPR signal from Aβ immobilized on the flow cells. Experiments were conducted in PBS (pH 7.4), and apoE isoforms were injected at a flow rate of 30 μL/min. Dissociation was followed in the same buffer for 6 min. After each run, the sensor chip was regenerated using 2 M guanidine-HCl, 10 mM Tris·HC1, pH 8.0, and was washed with running buffer for 10 min before the next injection. Data analysis was performed using BIAevaluation software (GE-BIAcore), and dissociation constants were calculated using a single-site binding model in GraphPad Prism Software. Data are based on two independent measurements using six different concentrations for each measurement. K_d values are presented as mean ± SD.

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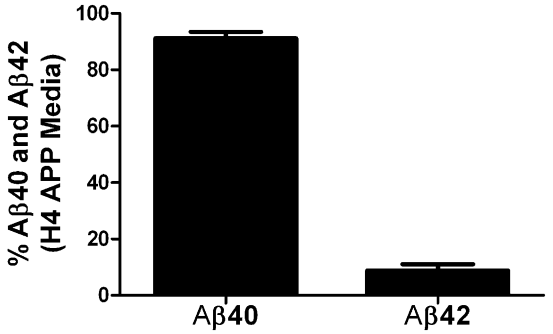

Fig. S1. H4 APP695ΔNL cells were incubated with serum-free Opti-MEM medium for 24 h. Then medium was collected, and the amounts of Aβ40 (2G3, anti–Aβ 35–40) and Aβ42 (21F12, anti–Aβ 33–42) were determined by ELISA using 3D6 (anti–Aβ 1–5) antibody as a coating antibody.

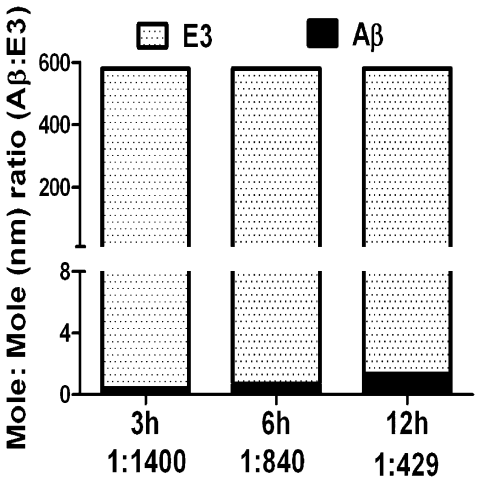

Fig. S2. The bar graph represents the moles of apoE3 and sAβ present in the region with a KBr density less than 1.15 g/cm^3. The mole:mole ratio was obtained from data presented in Fig. 1*F*.

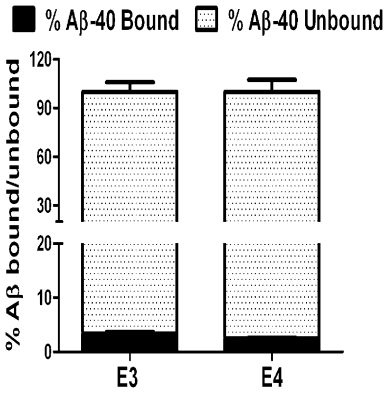

Fig. S3. The bar graph represents the percentage of soluble Aβ (sAβ) present in the region with a KBr density less than 1.15 g/cm^3 (percent Aβ bound) and in the region with a KBr density greater than 1.15 g/cm^3 (percent Aβ unbound) when synthetic Aβ (100 ng/mL) and 20 μg of lipidated rapoE3, and rapoE4 particles (apoE:phospholipid:cholesterol ratio, 1:50:10) was incubated for 6 h (Aβ:ApoE molar ratio of 1:25). Significance was assessed using a Student *t* test ($n = 3$ per group).

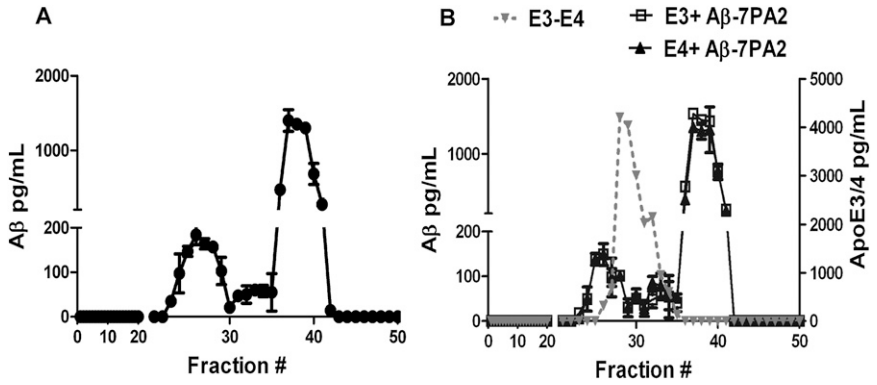

Fig. S4. (*A*) 7PA2 CM (sAβ,100 ng/mL) was concentrated with a 3-kDa cutoff concentrator and subjected to SEC with a Superdex 75 column. Fractions were analyzed for $A\beta_{1-x}$ by ELISA as described in *SI Materials and Methods*. (*B*) 7PA2 cell CM (sAβ, 100 ng/mL) was incubated with 10 μg of astrocyte-derived apoE3 and apoE4 for 6 h and subjected to SEC. Fractions were analyzed for apoE and Aβ by ELISA as described in *SI Materials and Methods*.

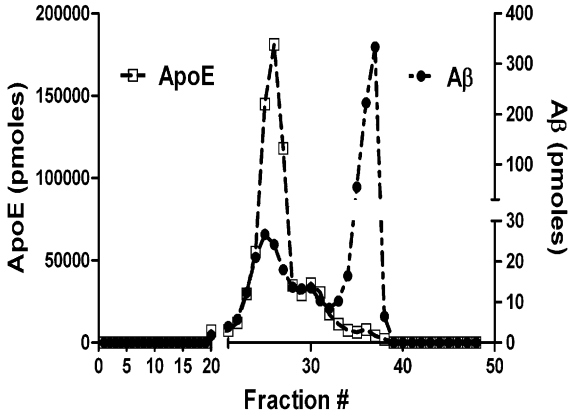

Fig. S5. Pooled CSF (2.0 mL) from human subjects was concentrated with a 3-kDa cutoff (0.5 mL) concentrator and subjected to SEC. Fractions were analyzed for apoE and Aβ by ELISA as described in *SI Materials and Methods* ($n = 2$).

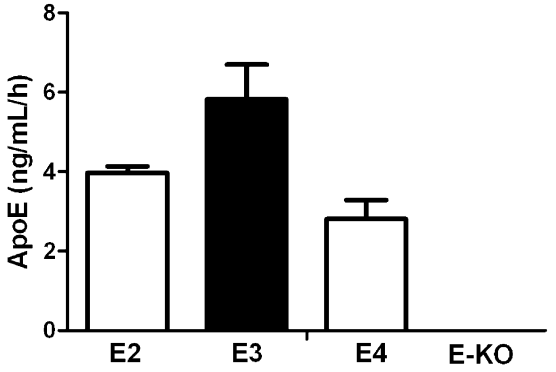

Fig. S6. To assess the levels of apoE secreted into cell-culture medium by apoE2-, apoE3-, and apoE4-expressing and apoE-KO astrocytes, the cells were incubated with serum-free growth medium, and the medium was collected after 12 h to assess apoE levels by ELISA as described in *SI Materials and Methods*. There was no significant difference in the level of apoE in the medium of cells secreting the different apoE isoforms.

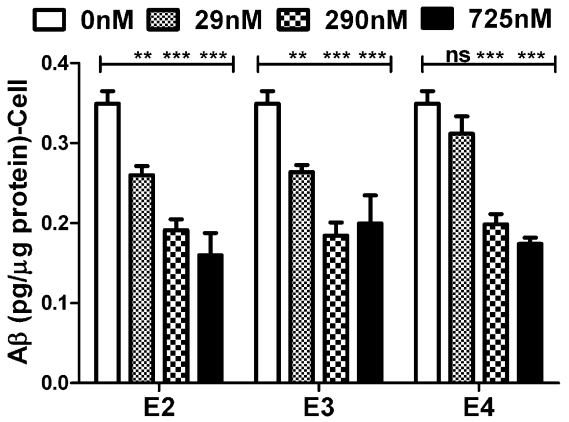

Fig. S7. To determine whether low lipidated apoE and Aβ compete for the same cellular uptake pathways, ApoE-KO astrocytes were incubated with CM from H4 APP695ΔNL cells (sAβ, 100 ng/mL) and the indicated concentrations of rapoE2, rapoE3, and rapoE4 (apoE:phospholipid:cholesterol ratio,1:5:1) for 12 h. The amount of Aβ in the cell lysate then was assessed by ELISA as described in *SI Materials and Methods*. Significance was assessed using one-way ANOVA followed by a Dunnet posttest (n = 4–6). Data are shown as mean ± SEM ($n \geq 4$). *$P < 0.05$, **$P < 0.005$, *** $P < 0.0005$; n.s., not significant.

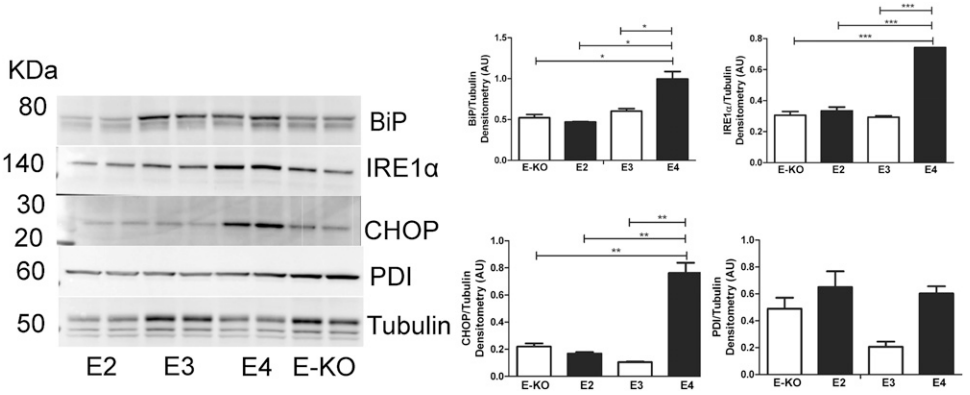

Fig. S8. To assess the role of apoE isoform on ER stress, astrocytes expressing apoE2, apoE3, or apoE4 and apoE-KO astrocytes were incubated with CM from H4APP cells (sAβ,100 ng/mL) for 12 h. (*Left*) The cells were collected, and Western blot analysis was performed for ER stress markers. (*Right*) Differences were assessed using one-way ANOVA followed by a Bonferroni posttest (n = 4). Data are shown as mean ± SEM (n = 4). *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.

Table S1. SPR analysis of interaction between apoE isoforms and synthetic Aβ

K_d, nM	Aβ40 1:50:10	Aβ42 1:50:10	Aβ40 1:5:1	Aβ42 1:5:1	Aβ40 NL	Aβ42 NL
rapoE2	262 ± 45	297 ± 41.4[ns]	96.5 ± 11*	99 ± 10[†]	118 ± 16[‡]	132 ± 13[¶]
rapoE3	190 ± 31.5	253 ± 33.3[ns]	101.2 ± 11*	97 ± 9.2[†]	99 ± 10.56[‡]	109 ± 9.3[¶]
rapoE4	266 ± 50	750 ± 96[§]	138.2 ± 16[ns]	327 ± 65[†]	159 ± 15.2[ns]	261 ± 42[¶]

SPR spectroscopy was used to measure the interaction between rapoE2, rapoE3, or rapoE4 and Aβ40 or Aβ42 in apoE:phospholipid:cholesterol concentrations of 1:50:10 or 1:5:1 and in nonlipidated (NL) Aβ40 or Aβ42. Aβ40 and Aβ42 were immobilized on the SPR chip, and various concentrations of rapoE2, rapoE3, and rapoE4 were flowed over the surface. To calculate the K_d, we plotted the resonance units as a function of rapoE2, rapoE3, and rapoE4 concentration. Significance was assessed using a Student's t test. Data are shown as mean ± SEM. n.s., not significant (n = 2).

*Compare E2/E3/E4 (1:50:10) vs. E2/E3/E4 (1:5:1) binding to Aβ40. $P < 0.05$.

[†]Compare E2/E3/E4 (1:50:10) vs. E2/E3/E4 (1:5:1) binding to d Aβ42. $P < 0.05$.

[‡]Compare E2/E3/E4 (1:50:10) vs. E2/E3/E4 (NL) binding to Aβ40. $P < 0.05$.

[§]Compare E3 (1:50:10) vs. E4 (1:50:10) binding to Aβ40 and Aβ42. $P < 0.05$.

[¶]Compare E2/E3/E4 (1:50:10) vs. E2/E3/E4 (NL) binding to Aβ42. $P < 0.05$.