Co-aggregation with Apolipoprotein E modulates the function of Amyloid-β in Alzheimer's disease

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Abstract

Isoforms of Apolipoprotein E (ApoE) determine our risk of developing late-onset Alzheimer's Disease (AD), but the mechanism underlying this link is poorly understood. In particular, the relevance of direct interactions between ApoE and Amyloid-β (Aβ) remains controversial. Here, single-molecule imaging shows that in the early stages of aggregation, all isoforms of ApoE associate with Aβ in large co-aggregates, but then fall away as fibrillation happens. Similar large co-aggregates exist in the brains of AD patients, accounting for around 50% of the mass of aggregated Aβ detected in the frontal cortices of homozygotes with the higher-risk *APOE4* gene. The cellular uptake and toxicity of these large co-aggregates are isoform-dependent, suggesting a mechanistic role for ApoE-Aβ interactions in AD.

Main text

Inherited variation in the sequence of Apolipoprotein E (ApoE) is the greatest genetic risk factor for late-onset Alzheimer's Disease (AD), the most prevalent form of dementia, accounting for ~95% of all AD cases(*1*). The *APOE* gene has three alleles: *APOE2*, *APOE3*, and *APOE4*. Compared to the most common *APOE3* form, *APOE2* is neuroprotective(*2*), while *APOE4* increases AD risk; *APOE4* homozygotes are 15-fold more susceptible to AD than *APOE3* homozygotes(*3*, *4*), and disease begins several years earlier in *APOE4* carriers than those with *APOE3* or *APOE2*(*3*).

Attempts to establish how ApoE influences AD risk have focused on its effect on the pathological Amyloid-beta (Aβ) peptide(*1*, *5*). Deposits of Aβ aggregates in the central nervous system (CNS) are a hallmark of AD(*6*). These aggregates induce synaptic and axonal damage as well as influence tau seeding and spreading, leading to neurodegeneration in AD(*7–10*). A set of compelling evidence suggests that ApoE4 enhances Aβ pathology(*5*). Carriers of *APOE4* have more Aβ deposits in their CNS than non-carriers(*1*, *11*), exhibit amyloid positivity earlier in life(*12*) and experience a faster-growing Aβ burden(*13*). The isoforms of the ApoE protein seem to differentially affect the levels of Aβ in the CNS, which may explain their differential AD risk profiles. Although observational studies show that ApoE influences the relationship between Aβ and cognitive decline in AD(*14–16*), it is not clear how ApoE affects Aβ aggregation and aggregate-induced neurotoxicity at the molecular level.

The accelerated Aβ plaque deposition in carriers of *APOE4* (*13*, *17*) has led to several hypotheses for ApoE4's molecular role in AD. One possibility is that ApoE4 promotes more aggregation of Aβ than the other isoforms by interacting directly with Aβ, when the two meet in the extracellular space. The discovery of co-deposited ApoE in AD amyloid plaques provided early circumstantial evidence for this idea(*18*, *19*), but biophysical studies are equivocal: ApoE can either speed up or slow down Aβ aggregation *in vitro*, depending on the conditions(*20*). Although targeting ApoE with an antibody can significantly reduce Aβ pathology in transgenic mouse models because some forms of ApoE are present in amyloid plaques (*18*, *19*), different studies have found that soluble forms of ApoE and Aβ may or may not associate significantly in brain tissue(*21*, *22*). The role of ApoE-Aβ interactions in Aβ clearance is also unclear: while ApoE might traffic Aβ out of the interstitial brain fluid(*17*, *23*), other work suggests that ApoE and Aβ instead compete for clearance-mediating receptors(*21*).

We began our study by investigating how ApoE interacts with Aβ along its aggregation pathway to fibrils. Aggregating Aβ comprises a dynamic, heterogeneous mixture of species with different sizes, shapes and properties, and small sub-populations may disproportionately contribute to AD(*24–26*). We reasoned that ApoE could influence AD risk by interacting with Aβ aggregates that are transient and/or rare, which might explain why previous attempts to study association by taking snapshots of the bulk mixture have painted an inconsistent picture. We therefore aggregated Aβ42 *in vitro* at a concentration (4 μM) that would give rise to fibrils, in the presence and absence of near-

physiological concentrations of each nonlipidated ApoE isoform (80 nM). We used nonlipidated ApoE because decreasing ApoE lipidation increases Aβ pathology(*27*), and the risk of developing AD(*22*), whereas lipidation of ApoE impedes Aβ fibril formation(*28*); nonlipidated ApoE is also a promising target for treating AD (*19*). Assaying fibril formation using ThT fluorescence confirmed that all reactions produced fibrils, albeit more slowly in the presence of ApoE (Fig. 1A, fig. S1).

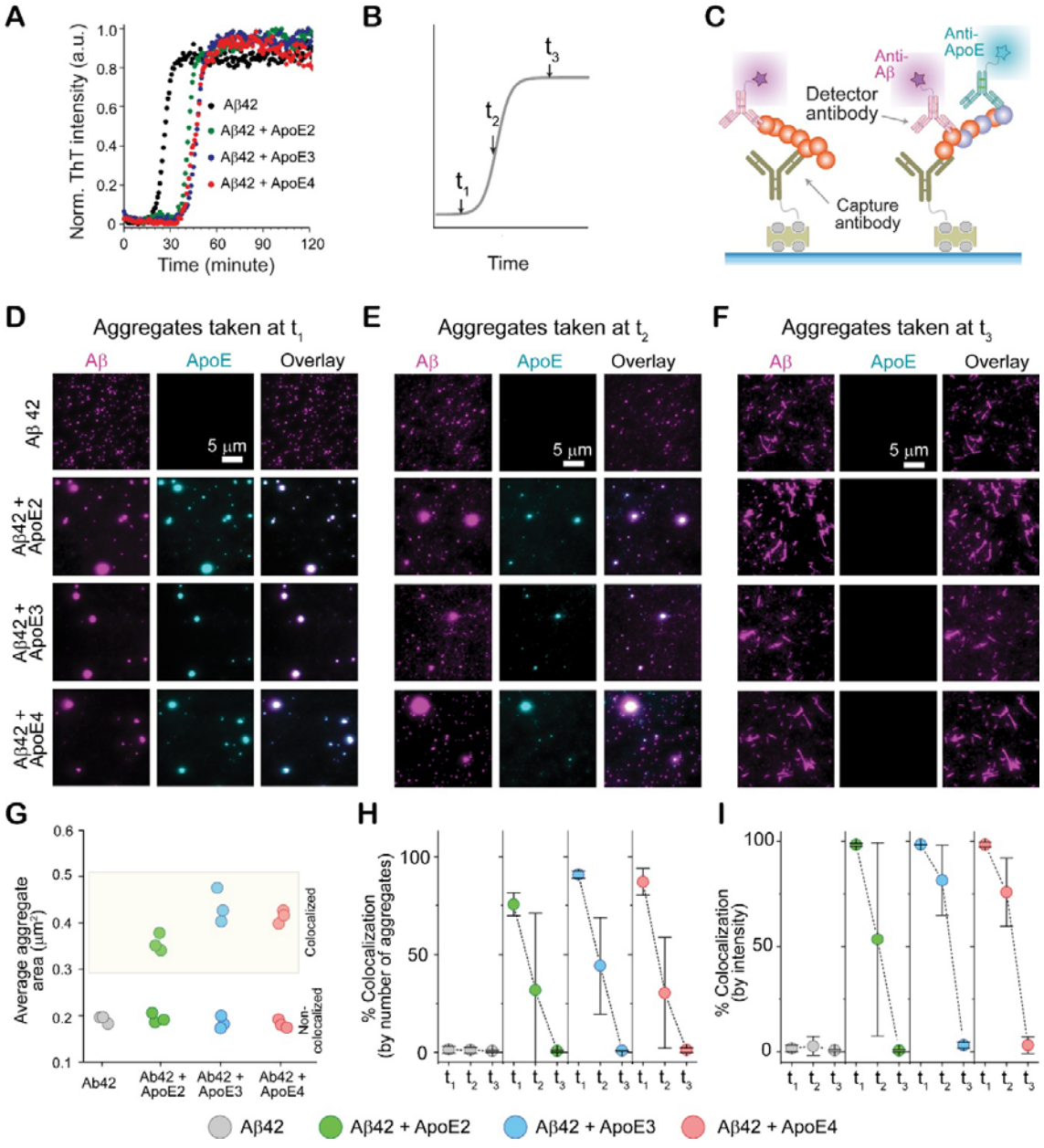

Figure 1. ApoE and Aβ transiently co-aggregate on the pathway to fibrils. (A) Aβ42 aggregation (4 μM) in the presence of different isoforms of ApoE (0 or 80 nM), monitored by ThT fluorescence (*n* = 3 independent replicates). **(B)** Schematic showing the time points at which samples were taken for further analysis. **(C)** Schematic of SiMPull assay for Aβ42 aggregates and Aβ-ApoE co-aggregates (1 μM Aβ monomer equivalents), using biotinylated 6E10 antibody for capture, and Alexa-Fluor-647-labeled 6E10 (500 pM) and Alexa-Fluor-488-

labeled EPR19392 (1 nM) antibodies for detection. **(D, E, F)** Two-color TIRF images of aggregates captured at t_1 **(D)**, t_2 **(E)** and t_3 **(F). (G)** Average sizes of colocalized and non-colocalized aggregates (in diffracted-limited imaging, minimum aggregate size can be resolved ~0.2 μm^2). **(H, I)** Colocalization between Aβ and ApoE at different time points, quantified by aggregate counting **(H)** and 6E10 fluorescence intensity **(I)**. Data are plotted as the mean and standard deviation of three technical replicates.

In order to best sample the heterogeneity within each aggregation pathway, we measured individual aggregates at different stages of the reaction. We imaged each reaction at the end of the lag phase (t_1), middle of the growth phase (t_2) and the plateau phase (t_3), using single-molecule pull-down (SiMPull) (Fig. 1B). In this assay (Fig. 1C), Aβ42 is captured using a surface-tethered 6E10 antibody and imaged using two-color total internal reflection fluorescence (TIRF), after adding primary detector antibodies for Aβ (Alexa-Fluor-647-labeled 6E10) and ApoE (Alexa-Fluor-488-labeled EPR19392) (fig. S2). Using the same monoclonal antibody to sandwich Aβ aggregates renders unreacted monomers undetectable because they only contain one epitope.

Characterizing individual aggregates rather than their ensemble average allowed us to extract properties of the heterogeneous population including size, shape and composition; aggregates containing both ApoE and Aβ42 are colocalized in both detection channels. These data (Fig. 1 D-F) revealed that ApoE and Aβ42 co-aggregate in the early stages of aggregation, but that co-aggregates disappear as the reaction reaches completion. At t_1 and t_2, ApoE formed large co-aggregates (diameter ~500-900 nm) with Aβ42 (Fig 1G), with no significant differences between isoforms in the extent of colocalization, whether based on the aggregate number (Fig. 1H), or intensity (Fig. 1I). These findings are independent of which antibody is used to detect ApoE (fig. S3), and no colocalization is observed when isotype-control detection antibodies are used (fig. S4), suggesting minimal contributions from non-specific binding. Using the intensity as a proxy for the amount of protein present in each aggregate suggests that 85-100% of aggregate mass at the end of the lag phase is in co-aggregates, which falls to ~30%-60% in the growth phase, and 0% by the plateau phase.

Our finding that ApoE forms large soluble co-aggregates with Aβ supports the idea that ApoE can stabilize soluble species formed early on in aggregation and thus inhibit Aβ fibrillation(*29*). Since ApoE is present in sub-stoichiometric amounts (Aβ: ApoE 50:1), sequestration of Aβ monomers is unlikely to account for our observations. ApoE more likely interacts with Aβ42 in its earliest stages of aggregation; ApoE did not associate with pre-formed Aβ42 aggregates (fig. S5). The high fluorescence intensities of these soluble co-aggregates indicate high effective protein concentrations, which may seem incompatible with decreased fibrillization rates. However, it has recently been shown that locally concentrating Aβ42 in condensates significantly slows its aggregation(*30*). The fact that fibrils have shed all associated ApoE might indicate that elongating heteronuclei is less energetically favorable than elongating homonuclei. Importantly, the lack of any isoform dependence means that co-aggregation alone cannot explain the *APOE* allele dependence of AD.

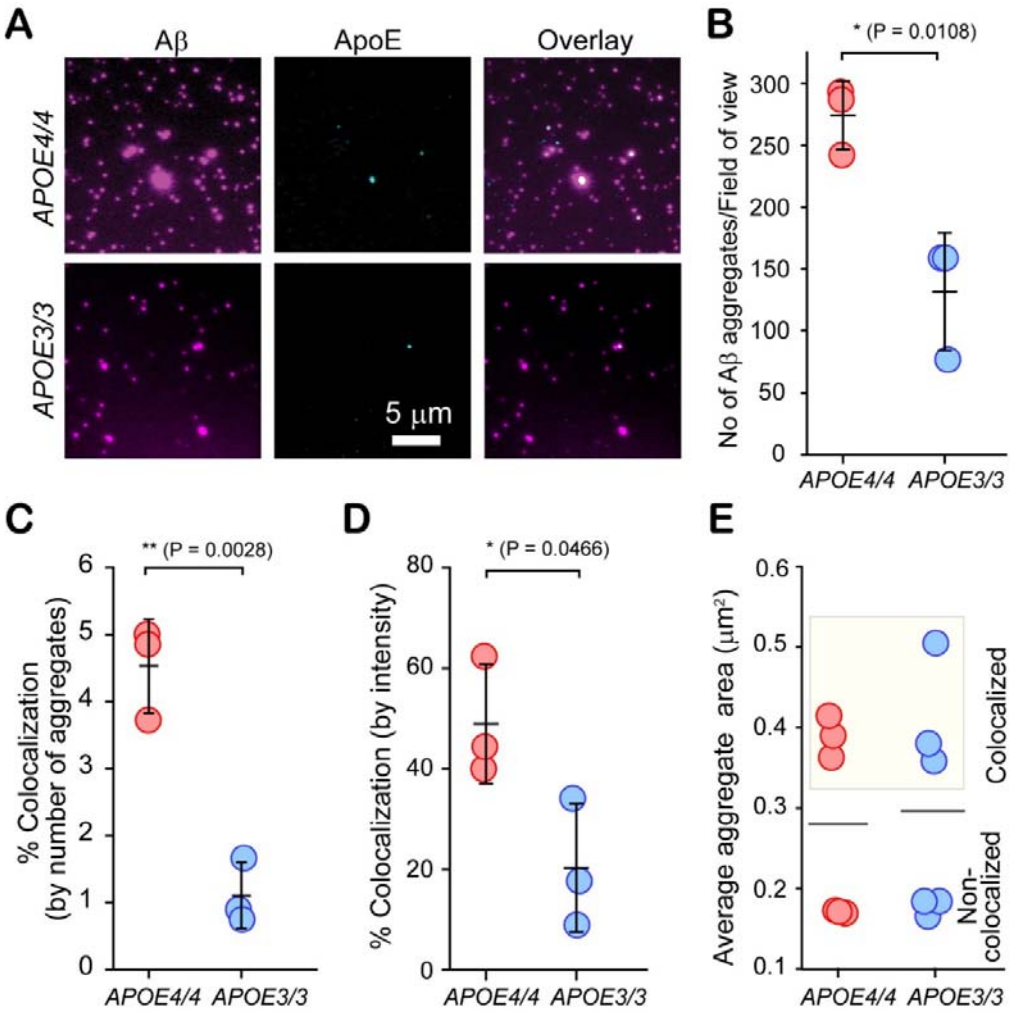

Figure 2. Aβ-ApoE co-aggregates form in human brain tissue, but their concentration is isoform dependent. (A) Two-color TIRF images of aggregates captured from frontal cortex extracts of homozygous *APOE4* and *APOE3* AD patients. **(B)** Total numbers of Aβ-containing species captured from *APOE4* and *APOE3* homozygotes. **(C, D)** Colocalization between Aβ and ApoE in extracts from *APOE4* and *APOE3* homozygotes, quantified by aggregate counting **(C)** and 6E10 fluorescence intensity **(D)**. **(E)** Sizes of ApoE-colocalized and non-colocalized Aβ aggregates in *APOE4* and *APOE3* homozygotes.

To determine whether these co-aggregates are disease relevant, we soaked postmortem frontal cortex tissue from six AD patients, using a method developed to gently extract soluble aggregates(*31*). We imaged the extracts from three homozygous *APOE4* and three homozygous *APOE3* AD patients using SiMPull (**Fig. 2A**). Despite the heterogeneous ages of the *ex vivo* plaques and aggregates, all samples yielded Aβ aggregates both with and without associated ApoE; the extracts from *APOE4* carriers contained more of both types of aggregates than extracts from *APOE3* carriers (Fig. 2B). This finding supports the previous report that *APOE4* carriers have more soluble Aβ aggregates than *APOE3* carriers(*32*). Unlike our *in vitro* aggregations - where the initiation of aggregation is synchronized for

all monomers - only 3-5% of Aβ aggregates in *APOE4* homozygotes and 1% of aggregates in *APOE3* homozygotes are co-aggregates (Fig. 2C). This difference probably results from the fact that Aβ and ApoE are replenished and cleared over years in the CNS, meaning that extracted aggregates reflect a broader sample of the reaction pathway, e.g. recently formed soluble aggregates alongside matured fibrils. Similar to co-aggregates formed *in vitro*, *ex vivo* co-aggregates are large (Fig. 2E) and contribute disproportionately to the fluorescence intensity. Using the intensity as a proxy for total amounts of protein suggests co-aggregates comprise 40-60% and 10-35% of the Aβ-aggregate mass in *APOE4* and *APOE3* carriers, respectively (Fig. 2D). These data therefore reveal a large isoform dependence in the accumulation of co-aggregates in AD brains.

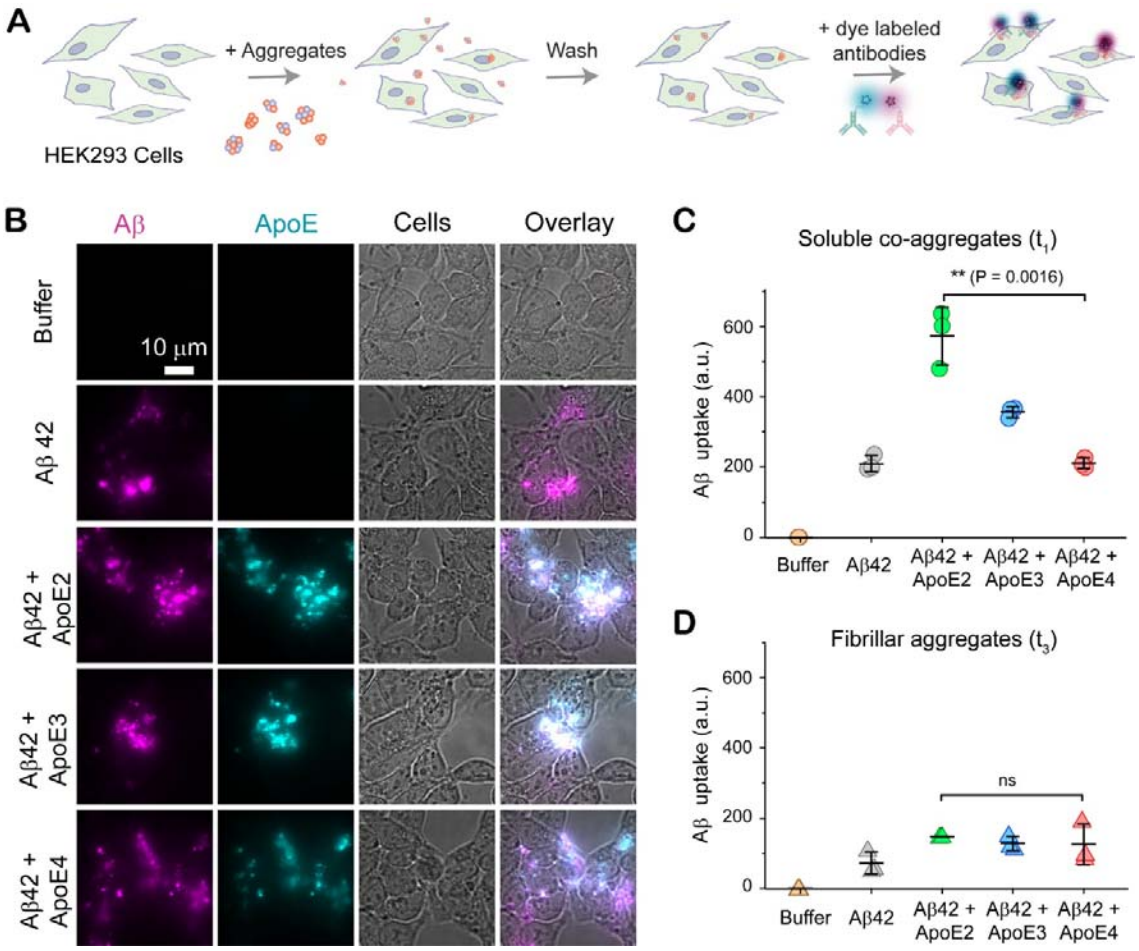

Figure 3. Association with ApoE enhances uptake of Aβ aggregates by cells in an isoform-dependent manner. (A) Schematic of cellular uptake assay **(B)** Two-color epifluorescence images showing uptake of Aβ42 (2 μM monomer equivalents) and ApoE (0 or 40 nM) from aggregation mixtures at t_1 (end of lag phase) by HEK293 cells, stained with Alexa-Fluor-647-labeled 6E10 antibody (Aβ) and Alexa-Fluor-488-labeled F-9 antibody (ApoE). **(C,D)** Uptake of Aβ42 from aggregation mixtures at t_1 (end of lag phase, where aggregates are mostly large soluble co-aggregates) **(C)** and t_3 (plateau phase, where aggregates are mostly Aβ-only fibrils) **(D)**.

Data were averaged over three biological replicates; error bars represent standard deviation and statistical significance was calculated using a two-sample t-test.

Given that all isoforms of ApoE had co-aggregated similarly with Aβ42, we next asked whether differential clearance might explain the isoform-dependent accumulation of ApoE-Aβ co-aggregates in AD brains. To isolate the role of co-aggregates, we compared the uptake of soluble aggregates from t_1 (~100% co-aggregates) and fibrillar aggregates from t_3 (~0% co-aggregates), which we quantified by immunostaining (Fig. 3A-D). HEK293 cells internalized 2-5-fold more soluble co-aggregates than fibrillar aggregates over a 24-hour period; co-aggregates with ApoE2 were removed significantly more efficiently from the media than co-aggregates with ApoE4, but this isoform dependence disappeared when cells were offered fibrillar aggregates, which do not contain ApoE. These data therefore support a role for ApoE-mediated clearance in the isoform dependence of AD risk.

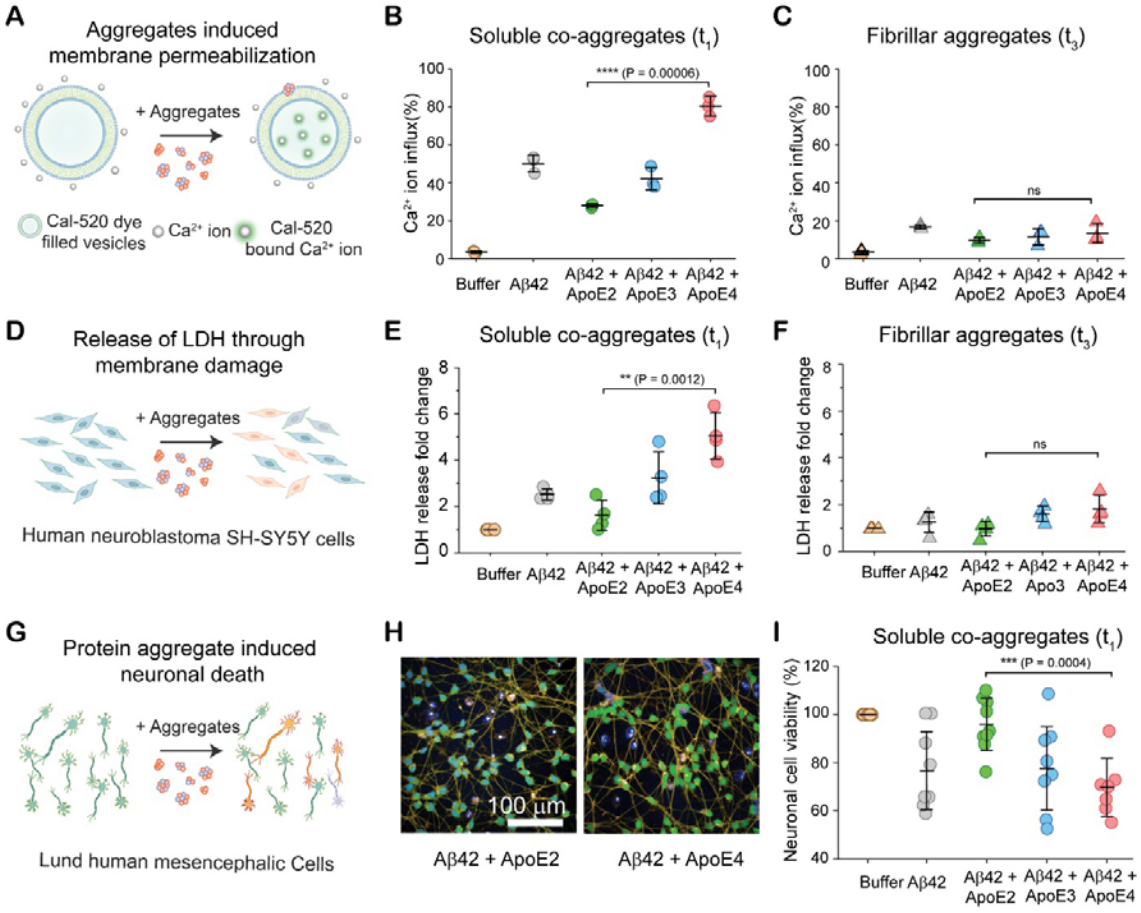

Figure 4. Isoforms of ApoE modulate the toxicity of Aβ aggregates differently. **(A-C)** Permeabilization of lipid bilayers by Aβ42 and Aβ-ApoE co-aggregates from aggregation mixtures at t_1 (end of lag phase, where aggregates are mostly large soluble co-aggregates) **(B)** and t_3 (plateau phase, where aggregates are mostly Aβ-only fibrils) **(C)** ([Aβ42] = 4 μM in monomer equivalents; [ApoE] = 0 or 80 nM). Ca^{2+} influx is referenced to the influx caused by the ionophore, ionomycin. **(D-F)** Neurotoxicity of Aβ42 and Aβ-ApoE co-aggregates from aggregation mixtures at t_1 **(E)** and t_3 **(F)** in human neuroblastoma SH-SY5Y cells, assayed by lactate

dehydrogenase (LDH) release ([Aβ42] = 2 μM in monomer equivalents; [ApoE] = 0 or 40 nM). **(G-I)** Schematic of the neuronal viability assay **(G)**, fluorescence images **(H)** of human LUHMES neuronal cells after incubation with Aβ-ApoE2 and Aβ-ApoE4 co-aggregates and neurotoxicity **(I)** of Aβ42 and Aβ-ApoE co-aggregates from aggregation mixtures at t_1 in LUHMES cells ([Aβ42] = 1 μM in monomer equivalents; [ApoE] = 0 or 20 nM).

Finally, we investigated the cytotoxicity of Aβ-ApoE co-aggregates. We measured this property of co-aggregates in three ways: i) their ability to permeabilize lipid membranes (Fig. 4A-C); ii) their toxicity to the SH-SY5Y human cell line, assayed by the release of lactate dehydrogenase (LDH) (Fig. 4D-F); iii) their effect on neuronal viability of Lund human mesencephalic (LUHMES) cells (Fig. 4G-I). Again, we compared soluble co-aggregates from t_1 to fibrillar aggregates from t_3 - which had shed ApoE - in order to isolate the role of co-aggregates. Each assay painted a similar picture: soluble aggregates were better at permeabilizing lipid bilayers and more toxic to cells than fibrillar aggregates, and among the soluble co-aggregates, toxicity followed the trend ApoE4>ApoE3>ApoE2.

This study identifies a potential role for large, soluble ApoE-Aβ co-aggregates in forging the link between *APOE* alleles and late-onset AD risk. Starting from the premise that ApoE could affect AD risk by interacting with Aβ aggregates that are either rare or transient, we sampled a broad cross-section of the aggregation equilibrium, showing that ApoE-Aβ co-aggregates are transient, but ubiquitous at the end of the lag phase of *in vitro* aggregation. All isoforms of ApoE co-aggregate similarly with Aβ *in vitro*, but we found more co-aggregates in the brains of *APOE4* homozygotes than *APOE3* homozygotes. This difference could result from isoform-dependent clearance, supported by the fact that uptake of co-aggregates follows the trend ApoE2>ApoE3>ApoE4. The cytotoxicity of co-aggregates follows the opposite pattern (i.e. ApoE4>ApoE3>ApoE2), suggesting that a combination of differential clearance and neurotoxicity of ApoE-Aβ co-aggregates connects *APOE* genotypes with amyloid loads in CNS, age of disease onset and rate of disease progression.

These findings suggest further avenues for investigating and treating late-onset AD. Establishing how the observed isoform-dependent uptake of large soluble ApoE-Aβ co-aggregates translates to glial cells, particularly microglia and astrocytes, will be important to understand how co-aggregates influence AD pathology. The fact that we found opposing trends in the isoform-dependence of internalization and membrane permeabilization of co-aggregates suggests uptake may be mainly receptor mediated. ApoE's influence on endocytosis is well studied, with roles for other AD risk factors such as TREM2(*33*) and *PICALM*(*34*), while LRP1 and VLDLR are putative receptors for the transport of ApoE-Aβ complexes(*23*). Validating specific receptor(s) which play a significant role in the internalization of large soluble co-aggregates could present opportunities to precisely target ApoE in AD; modulating the formation or uptake of large, soluble co-aggregates in an isoform-specific way could eliminate the AD-specific role of ApoE without impeding its essential functions in lipid transport.

Author Contributions

R.T.R. and S.D. did the SiMPull experiments. R.T.R. modified antibodies and coated coverslips for SiMPull. Z.X. and S.D. aggregated proteins. The cellular uptake assay was carried out by P.T., E.D., E.Z., S.D. and H.H. prepared soaked-brain extracts, Z.X., E.P., and S.D. did membrane permeabilization and LDH assays, T.L. did the neuronal viability assay, H.J., H.Z., Y.L.J.L., Y.Z., and J.D. helped with antibody modifications, slide preparation or microscopy. Data analysis and statistics were developed, carried out by Z.X. and interpreted by Z.X. and S.D. R.T.R., H.M., P.T., D.C.C., D.M.H., D.K. and S.D. supervised the project. The initial draft of the paper was written by R.T.R., D.K. and S.D.; all other authors provided feedback and contributed in editing the manuscript into its final form. D.K. and S.D. designed and conceived the study. All authors read and approved the manuscript.

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Competing interests

D.M.H. is as an inventor on a patent licensed by Washington University to C2N Diagnostics on the therapeutic use of anti-tau antibodies. D.M.H. co-founded and is on the scientific advisory board of C2N Diagnostics. C2N Diagnostics has licensed certain anti-tau antibodies to AbbVie for therapeutic development. D.M.H. is on the scientific advisory board of Denali and consults for Genentech, Merck, Eli Lilly, and Cajal Neuroscience. The lab of D.M.H. receives research grants from the National Institutes of Health, Cure Alzheimer’s Fund, Tau Consortium, the JPB Foundation, Good Ventures, C2N Diagnostics, NextCure, and Denali. D.C.C. holds stock in AstraZeneca. All the other authors declare no conflicts of interest

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