Increased Aβ_{42}-α7-like nicotinic acetylcholine receptor complex level in lymphocytes is associated with apolipoprotein E4-driven Alzheimer’s disease pathogenesis

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Abstract

Background: The apolipoprotein E ε4 (APOE4) genotype is a prominent late-onset Alzheimer’s disease (AD) risk factor. ApoE4 disrupts memory function in rodents and may contribute to both plaque and tangle formation.

Methods: Coimmunoprecipitation and Western blot detection were used to determine: 1) the effects of select fragments from the apoE low-density lipoprotein (LDL) binding domain and recombinant apoE subtypes on amyloid beta (Aβ)_{42}-α7 nicotinic acetylcholine receptor (α7nAChR) interaction and tau phosphorylation in rodent brain synaptosomes; and 2) the level of Aβ_{42}-α7nAChR complexes in matched controls and patients with mild cognitive impairment (MCI) and dementia due to AD with known APOE genotypes.

Results: In an ex vivo study using rodent synaptosomes, apoE_{141–148} of the apoE promotes Aβ_{42}-α7nAChR association and Aβ_{42}-induced α7nAChR-dependent tau phosphorylation. In a single-blind study, we examined lymphocytes isolated from control subjects, patients with MCI and dementia due to AD with known APOE genotypes, sampled at two time points (1 year apart). APOE ε4 genotype was closely correlated with heightened Aβ_{42}-α7nAChR complex levels and with blunted exogenous Aβ_{42} effects in lymphocytes derived from AD and MCI due to AD cases. Similarly, plasma from APOE ε4 carriers enhanced the Aβ_{42}-induced Aβ_{42}-α7nAChR association in rat cortical synaptosomes. The progression of cognitive decline in APOE ε4 carriers correlated with higher levels of Aβ_{42}-α7nAChR complexes in lymphocytes and greater enhancement by their plasma of Aβ_{42}-induced Aβ_{42}-α7nAChR association in rat cortical synaptosomes.

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Conclusions: Our data suggest that increased lymphocyte $\text{A}_\beta_{42}$-a7nAChR-like complexes may indicate the presence of AD pathology especially in APOE e4 carriers. We show that apoE, especially apoE4, promotes $\text{A}_\beta_{42}$-a7nAChR interaction and $\text{A}_\beta_{42}$-induced a7nAChR-dependent tau phosphorylation via its apoE141-148 domain. These apoE-mediated effects may contribute to the APOE e4-driven neurodysfunction and AD pathologies.

Keywords: Alzheimer’s disease, Mild cognitive impairment, $\beta$-Amyloid, Apolipoprotein E, a7 Nicotinic acetylcholine receptor, tau phosphorylation, Synaptosome, Lymphocyte, Biomarker

Background
The severity of neurodegeneration in Alzheimer’s disease (AD) correlates with the soluble amyloid beta ($\text{A}_\beta$) level in the brain [1]. $\text{A}_\beta$ binds selectively and with high affinity to neuronal a7 nicotinic acetylcholine receptors (a7nAChRs), leading to intraneuronal $\text{A}_\beta_{42}$ accumulation, tau phosphorylation, and cholinergic dysfunction [2–5]. Therefore, chronic perturbation of the a7nAChRs by $\text{A}_\beta$ may contribute to neuronal dysfunctions and neurodegeneration leading to the formation of $\text{A}_\beta$-rich plaque and neurofibrillary pathologies, which may be reduced by treatments that disrupt the $\text{A}_\beta_{42}$-a7nAChR interaction. This hypothesis is supported by data showing that S 24795, an $\alpha$7nAChR partial agonist, blocks the $\text{A}_\beta_{42}$-a7nAChR interaction, $\text{A}_\beta_{42}$ internalization into neuronal cells, and $\text{A}_\beta_{42}$-induced tau phosphorylation [4, 5]. The critical role of a7nAChR in the Aβ-driven AD pathogenesis and cognitive deficits is further substantiated by the report showing that deletion of the a7nAChR gene enhances cognitive deficits and synaptic pathology in a mouse model of AD [6]. Despite evidence of increased $\text{A}_\beta_{42}$-a7nAChR complex levels in lymphocytes from AD subjects [7], it remains ambiguous whether an increased $\text{A}_\beta_{42}$-a7nAChR complex level in lymphocytes may be a reliable AD biomarker. It is also unknown whether an increase in $\text{A}_\beta_{42}$-a7nAChR complexes is related to the apolipoprotein E (APOE) genotype, especially the e4 subclass that is regarded as a prominent genetic risk factor for AD [8].

ApoE regulates lipid metabolism and cholesterol transport in the brain. Among three apoE isoforms, apoE4 is the least metabolically stable and is a recognized risk factor for developing both familial and late-onset sporadic AD by promoting various neuropathological effects [9, 10]. Proteolytic fragments of apoE are elevated in AD brains [11] and some synthetic apoE fragments are neurotoxic [12, 13]. In a postmortem brain study, apoE4 was strongly correlated with vascular $\text{A}_\beta$ deposition and $\text{A}_\beta$ plaque density [14]. Biochemical, cell biological, and transgenic animal studies have indicated that apoE4 can promote AD pathogenesis by altering $\text{A}_\beta$ deposition and clearance to increase intraneuronal $\text{A}_\beta$ accumulation and plaque formation [15–19]. ApoE negatively affects the redox system [20], signaling cascades and Ca$^{2+}$ homeostasis in neurons [21, 22] as well as cytoskeletal structure and function [23, 24], but it enhances tau phosphorylation and consequent formation of neurofibrillary tangles (NFTs) [25–28]. However, the underlying mechanisms responsible for these apoE4-mediated deteriorating effects and the cause-effect relationships remain largely unclear.

More recently, apoE low-density lipoprotein (LDL) receptor binding domain-containing peptide fragments were shown to inhibit $\alpha$7nAChRs by interacting directly with the receptors [1, 2, 3] and $\text{A}_\beta_{42}$-a7nAChRs ligands and $\text{A}_\beta_{12-28}$, the a7nAChR binding domain of $\text{A}_\beta_{42}$, all reduce the $\text{A}_\beta_{42}$-a7nAChR association [3, 32, 33], and $\text{A}_\beta_{42}$ promotes tau phosphorylation via activating a7nAChRs [3, 5, 7]. We therefore examined the effects of these apoE fragments, and more importantly the apoE subtypes, on the $\text{A}_\beta_{42}$-a7nAChR interaction and on the consequent apoE4-mediated deteriorating effects and the cause-effect relationships remain largely unclear.

Since APOE e4 is a prominent late-onset AD risk factor, the $\text{A}_\beta_{42}$-a7nAChR complexes in lymphocytes derived from patients enrolled in the CL2-NEURO-003 study (ROSAS cohort) [34] with diverse APOE genotypes who gave blood samples at two time-points at least 1 year apart were examined to determine whether $\text{A}_\beta_{42}$-a7nAChR complexes in lymphocytes are correlated with APOE genotype (APOE e4 specifically). Our results indicate that apoE4 increases the abundance of $\text{A}_\beta_{42}$-a7nAChR complexes in the brain and lymphocytes. More importantly, we show that exogenous $\text{A}_\beta_{42}$ increases $\text{A}_\beta_{42}$-a7nAChR complex levels in lymphocytes of controls and subjects with mild cognitive impairment (MCI) to the heightened levels of AD lymphocytes. Hence, we explored whether the elevated $\text{A}_\beta_{42}$-a7nAChR complex levels and the magnitude of reduction by exogenous $\text{A}_\beta_{42}$ in promoting the $\text{A}_\beta_{42}$-a7nAChR association (reflected by $+\text{A}_\beta_{42}$/$\text{A}_\beta_{42}$ ratios) may be used as AD diagnostic biomarkers that depict the severity of AD pathologies.

Methods
Materials and chemicals
HISTOPAQUE-1077, Leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, soybean trypsin inhibitor, NaF, sodium vanadate, $\beta$-glycerophosphate, 2-mercaptoethanol, NMDA, glycine, Tween-20, and NP-40 were all purchased from Sigma. $\text{A}_\beta_{1-42}$ was obtained from Invitrogen. Biotinated $\text{A}_\beta_{1-42}$ and FITC-conjugated $\text{A}_\beta_{1-42}$
were obtained from Anaspec (San Jose, CA, USA). Anti-α7nAChR (SC-5544, SC-58607), CHRFAM7A (SC-133458), -actin (SC-7210) and -β-actin (SC-47778) were all purchased from Santa Cruz biotechnology. Anti-Aβ42 antibody (Ab5078P) was purchased from EMD Millipore. Reacti-Bind™ NeutrAvidin™ High binding capacity coated 96-well plates, covalently conjugated protein A/G-agarose beads, Pierce cell surface protein isolation kit, antigen elution buffer, and chemiluminescent reagents were purchased from Pierce Thermo Scientific. Recombinant human apoE2 (#350-12), apoE3 (#350-02), and apoE4 (#350-04) that produced in E. coli (>90% purity) were purchased from Peprotech. Aβ1–42 peptide (trifluoroacetic acid; TFA salt) was dissolved in 50 mM Tris, pH 9.0 containing 10% dimethyl sulfoxide (DMSO) and stored at −80 °C. Biotinated Aβ1–42 and fluorescein isothiocyanate (FITC)-conjugated Aβ1–42, both ammonium salts, were dissolved in 50 mM Tris, pH 8.0 containing 10% DMSO and stored at −80 °C. All test agents were made fresh according to the manufacturer’s recommendation. If DMSO was used as the solvent, the highest DMSO concentration in the incubation medium was 1%.

**LDL receptor binding domain of apoE**

Six apoE LDL receptor binding-domain-containing peptide fragments that showed differential α7nAChRs inhibition [29–31] were synthesized and dissolved in 10% DMSO containing 50 mM Tris HCl, pH 8.8. These peptides were amide-capped at the carboxyl terminus and acetylated at the amino terminus, except for apoE133–140 which has a free amino terminus.

apoE133–140: LRVRLASHLRKLRKRL
apoE133–140 (K → L): LRVRLASHLRKLRLL
apoE141–148 scrambled: RLKKLRKRL
apoE133–140L: LRVRLASHLRKLRKRL
apoE141–148: LRVRLASHLRKLRKRL
apoE141–148 (K → E): LRVRLASHLRKLRML

**Animals**

Eight to 10-week-old male Sprague-Dawley rats from Taconic (Germantown, NY, USA) were maintained on a 12-h light/dark cycle with food and water ad libitum. Rats were rapidly decapitated and brain frontal cortices (buffy coats) were extracted on ice immediately.

All animal procedures comply with the National Institutes of Health Guide for Care Use of Laboratory Animals and were approved by the City College of New York Animal Care and Use Committee (IACUC), Protocol No. 836.1.

**Clinical samples**

AD and MCI patients as well as control subjects were selected from the population of the ROSAS cohort (CL2-NEURO-003 study, sponsored by SERVIER laboratories, performed at Alzheimer’s Disease Research and Clinical Center, Inserm U1027, Toulouse University Hospital, Toulouse, France). Human participants and their informed caregiver took part in the study on a voluntary basis, and they gave their written informed consent at selection. The ethics committee of Toulouse University Hospital approved the study protocol and all its amendments (registration number DGS 20060500).

Four hundred and eight (408) subjects aged 65 years and older were enrolled in the study, and they were divided into three groups and followed for 4 years: 110 normal controls (Mini-Mental State Examination (MMSE) ≥26, Clinical Dementia Rating (CDR) = 0); 100 patients with memory impairment without dementia (MCI; MMSE ≥24, CDR = 0.5); 100 patients with memory impairment (Rey Auditory Verbal Learning Test (RAVLT), but not Diagnostic and Statistical Manual of Mental Disorders, version IV (DSM IV) criteria for Dementia; 196 patients with dementia of the Alzheimer’s type (AD; 12 ≤ MMSE ≤ 26, CDR ≥0.5, DSM IV criteria). Participants and their informed caregivers participated on a voluntary basis, and gave their written informed consent at inclusion. The ethics committee of Toulouse University Hospital approved the study protocol. For details, see de Mauleon et al. [34].

**Selection of APOE genotype subpopulations**

We selected patients and their matched controls from four of the most represented APOE genotypes: APOE ε2/ε3, APOE ε3/ε3, APOE ε3/ε4, and APOE ε4/ε4. Within each of the four APOE genotypes selected, AD and MCI patients as well as controls must have at least two sets of plasma and blood ‘buffy coat’ samples taken 1 year apart (e.g., at visit M0 and M12 or M12 and M24 that are designated as visit 1 and visit 2). The potential study subjects were then selected and matched according to their age, gender, and level of education using a SAS® iterative algorithm. In each triad/pair selected, the absolute difference between the youngest and the oldest must not exceed 5 years.

A total of 86 subjects including 24 controls (11 females/13 males, 77.91 ± 0.86 years), 30 MCI (19 females/11 males, 77.53 ± 0.84 years), and 32 AD (18 females/14 males, 77.38 ± 0.80 years) patients, paired per age, level of education, and gender for the four most represented genotypes. The APOE ε2/ε3 group has 5 AD (3 females/2 males, 78.20 ± 2.62 years), 3 MCI (1 female/2 males, 81.67 ± 1.21 years), and 5 control (1 female/4 males, 78.40 ± 3.21 years) subjects. The ApoE3/E3 group has 10 AD (7 females/3 males, 79.00 ± 1.08 years), 10 MCI (6 females/4 males, 79.00 ± 1.08 years), and 10 control (7 females/3 males, 79.00 ± 1.08 years) subjects, the ApoE3/E4 group has 10 AD (5 females/5 males, 76.80 ± 1.37 years), 10 MCI (4 females/6 males, 77.00 ± 1.30 years), and 9 control
was terminated by adding ice-cold Ca²⁺-free K-R containing (in mM): 25 HEPES, pH 7.4, 118 NaCl, 4.8 KCl, 25 NaHCO₃, 1.3 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 10 glucose, 0.1 ascorbic acid, and a mixture of protease and protein phosphatase inhibitors (Roche Diagnostics) that had been aerated for 10 min with 95% O₂/5% CO₂. The protein concentration was determined using the Bradford method (Bio-Rad).

Preparation of the synaptosomes
Rats were sedated by CO₂ inhalation and killed by decapitation. FCXs were immediately dissected, homogenized, and processed immediately after harvesting to obtain synaptosomes (P2 fraction), as described previously [3] for neuropharmacological assessments. Synaptosomes were washed twice and suspended in 2 ml ice-cold oxygenated Krebs-Ringer (K-R) containing (in mM): 25 HEPES, pH 7.4, 118 NaCl, 4.8 KCl, 25 NaHCO₃, 1.3 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 10 glucose, 0.1 ascorbic acid, and a mixture of protease and protein phosphatase inhibitors (Roche Diagnostics) that had been aerated for 10 min with 95% O₂/5% CO₂. The protein concentration was determined using the Bradford method (Bio-Rad).

Preparation of the lymphocytes
Lymphocytes were prepared from blood ‘buffy coat’ samples using Histopaque 1077 (Sigma), according to the manufacturer’s instruction [7]. Briefly, blood ‘buffy coat’ (approximately 250 μl) were layered onto 250 μl HISTOPAQUE-1077 at 25 °C. The entire contents were centrifuged at 400 x g for 30 min at 25 °C to obtain the lymphocyte-free plasma (top layer) and opaque interphase containing lymphocytes. The lymphocytes were mixed with 1 ml of oxygenized K-R and then centrifuged at 250 x g for 10 min twice. The resultant lymphocyte pellet was resuspended in 250 μl oxygenized K-R and used as the tissue source for the measurement of the Aβ42-α7nAChR complex level. The protein contents of the lymphocyte suspension were estimated using the Bradford method (Bio-Rad).

Ex vivo Aβ42 treatment and determination of Aβ42-α7nAChR association
To test the effect of the ApoE subtype on the Aβ42-α7nAChR interaction, rat cortical synaptosomes (200 μg) were incubated either simultaneously at 37 °C with 1 μl Aβ42 and 0.01–100 μM of apoE fragments, or with apoE isoforms for 10 min and then 30 min following the addition of 0.1 μM Aβ42. To assess the impact of ApoE in plasma from human subjects as a bioassay, 200 μg of rat cortical synaptosomes were incubated at 37 °C with K-R, 0.1 μM Aβ42 or 0.1 μM Aβ42 + 25 μl of plasma for 30 min. In a separate set of experiments, human lymphocytes (200 μg) were incubated at 37 °C with K-R or 0.1 μM Aβ42 for 30 min (total incubation volume: 250 μl). The reaction was terminated by adding ice-cold Ca²⁺-free K-R containing protease and protein phosphatase inhibitors and centrifuged. The obtained synaptosomes or lymphocytes were homogenized in 25 μl ice-cold immunoprecipitation buffer containing 25 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.2% 2-mercaptoethanol, and protease and protein phosphatase inhibitors by sonication for 10 s on ice and solubilized by nonionic detergents: 0.5% NP-40/0.2% Na cholate/0.5% digitonin for 60 min (4 °C) with end-to-end rotation. The obtained lysate was cleared by centrifugation at 20,000 x g for 30 min (4 °C) and the resultant supernatant (0.25 ml) was diluted fourfold with 0.75 ml immunoprecipitation buffer. The Aβ42-α7nAChR complexes were immunoprecipitated with immobilized anti-Aβ42 antibodies on protein A-conjugated agarose beads. The resultant immunocomplexes were pelleted by centrifugation (4 °C), washed three times with ice-cold phosphate-buffered saline (PBS), pH 7.2, containing 0.1% Triton X-100, and centrifuged. The resultant immunocomplexes were solubilized by boiling for 5 min in 10 μl of 4X PAGE sample preparation buffer (62.5 mM Tris-HCl, pH 6.8; 10% glycerol, 2% SDS; 5% 2-mercaptoethanol, 0.01% bromophenol blue) and centrifuged to remove antibody-protein A/G agarose beads. The contents of α7nAChRs and actin were determined by Western blotting with the level of actin serving as the indicator of immunoprecipitation efficiency and gel loading [4, 5].

Determination of CHRFAM7A-α7nAChR association in membranes of lymphocytes
To assess the association of CHRFAM7A and α7nAChR on the lymphocyte membranes, lymphocytes (200 μg) obtained from ROSAS cohort were ruptured by sonication on ice in 250 μl hypotonic lysis buffer containing (in mM): 25 HEPES, pH 7.4, 118 NaCl, 0.48 KCl, 25 NaHCO₃, 0.13 CaCl₂, 0.12 MgSO₄, 0.12 KH₂PO₄, and a mixture of protease and protein phosphatase inhibitors. Following centrifugation at 50,000 x g for 30 min at 4 °C, the resultant lymphocytic cell membranes were homogenized by sonication on ice and solubilized by nonionic detergents: 0.5% NP-40/0.2% Na cholate/0.5% digitonin for 60 min (4 °C) with end-to-end rotation. The resultant lysates were cleared of debris by centrifugation at 20,000 x g for 30 min (4 °C) and the resultant supernatant (0.25 ml) was diluted fourfold with 0.75 ml immunoprecipitation buffer. The Aβ42-α7nAChR complexes were pelleted by centrifugation (4 °C), washed three times with ice-cold 0.1% NP-40 containing PBS, and centrifuged. The resultant immunocomplexes were solubilized by boiling for 5 min in 100 μl SDS-PAGE sample preparation buffer and then centrifuged to remove antibody-protein A agarose beads. The abundance of α7nAChRs in the anti-CHRFAM7A immunoprecipitate
was determined by Western blotting with anti-α7nAChR (SC-58607). The blot was then stripped, blocked with 10% nonfat milk containing 0.1% PBST for 1 h and incubated with anti-CHRFAM7A overnight at 4 °C to validate equal efficiency of the immunoprecipitation and gel loading.

Western blot analysis
Solubilized immunoprecipitates size-fractionated by 10% or 10–16% SDS-PAGE was electrophoretically transferred to nitrocellulose membranes. The membranes were washed with PBS three times and blocked overnight (4 °C) with 10% milk in 0.1% Tween-20-containing PBS (PBST). The membranes were washed with 0.1% PBST three times, incubated at 25 °C for 2 h or at 4 °C overnight with 1:500–1:1000 dilutions of selected antibodies including (α7nAChR (SC-58607), β-actin (SC-47778), and CHRFAM7A (SC-133458). After three 0.1% PBST washes, membranes were incubated for 1 h with anti-species IgG-HRP (1:5000–1:1000 dilutions) and threonine181-tau (pT181tau), threonine231-tau (pT231tau), and threonine–serine202-tau (pT202tau) contents were determined by Western blotting (Pierce-Thermo).

In vitro assessment of Aβ42-α7nAChR and Aβ42–Aβ42 interaction
The effect of apoE fragments and ApoE isoform on Aβ42-α7nAChR interaction was measured in vitro with nM biotinated α7nAChRs trapped on streptavidin-coated plate (Reacti-Bind™ NeutrAvidin™ High binding capacity coated 96-well plate; Pierce). Biotinylation of the cell surface proteins was performed using the Pierce cell surface protein isolation kit according to the manufacturer’s protocol. Briefly, T75 cm² dishes of 95% confluent SK-N-MC cells were quickly washed with ice-cold PBS. Biotinylation of the cell surface proteins was performed using sulfo-NHS-SS-Biotin. Following termination of the reaction, cells were scraped into PBS and collected by centrifugation. The cells were then lysed by brief sonication and centrifuged to obtain cell membranes. The resultant cell membranes were solubilized using 0.5% NP-40/0.2% sodium cholate/0.5% digitonin. The biotinylated α7nAChRs were isolated by immunoaffinity column with immobilized anti-α7nAChR antibodies. The plate was washed, blocked with 20% superblock (Pierce-Thermo), and incubated with K-R or 0.01–100 μM apoE fragment for 10 min followed by 60 min with 20 nM FITC-tagged Aβ42 at 30 °C. The plate was washed extensively and the residual FITC–Aβ42 signals were determined by multimode plate reader (DTX880; Beckman).

The effect of apoE fragments on Aβ42-α7nAChR interaction was measured in vitro with 2 nM biotinated Aβ42 trapped on streptavidin-coated 96-well plate, washed, and incubated with 0.01–100 μM of apoE fragments for 10 min prior to incubation with 20 nM FITC-tagged Aβ42 for 60 min at 30 °C. The plate was then washed five times with 50 mM Tris HCl, pH 7.5. The FITC–Aβ42 signals were detected using a multi-mode plate reader (DTX-880). Negligible FITC–Aβ42 was noted when either biotinated Aβ42 peptides or α7nAChRs were omitted.

Ex vivo determination of Aβ42-induced tau phosphorylation
The effect of apoE fragments on Aβ42-induced tau phosphorylation was examined using experimental procedure described previously [3, 5, 7]. Briefly, well-washed rat FCX synaptosomes (500 μg) were incubated in oxygenated K-R with 0.01–100 μM apoE fragment and/or 0.1 μM Aβ42 at 37 °C for 30 min. The total tau proteins were immunoprecipitated with anti-tau and the phosphorylated serine202-tau, threonine202-tau, threonine231-tau (pT231tau), and threonine–serine202-tau (pT202tau) contents were determined by Western blotting (Pierce-Thermo).

Statistical analyses
All data are presented as mean ± standard error from the mean (SEM). Treatment effects were evaluated by analysis of variance (ANOVA). Specifically, the apoE fragment and subtype effects of the Aβ42-α7nAChR association and tau phosphorylation in animal experiments were evaluated using one-way ANOVAs followed by Newman-Keul’s for multiple comparisons.

To analyze the biochemical data in the human studies, a mixed linear model was used (with pairing identifier as a random effect) in order to test paired differences among the three diagnostic groups as well as among the four ApoE genotypes. P values were corrected for multiple testing using the Dunnett’s approach. The threshold for significance was p < 0.05.

Correlations between criteria were evaluated using the Spearman correlation coefficient (with 95% confidence interval). SAS 9.2 and R 3.1.2 software were used to perform these analyses.

Results
Selective apoE LDL receptor binding domain fragments enhance the Aβ42-α7nAChR association
To evaluate the effect of apoE LDL receptor binding domain fragments on the Aβ42-α7nAChR association, rat FCX synaptosomes were incubated with 0.1–100 μM apoE LDL receptor binding domain fragments either 10 min prior to, or simultaneously with, 0.1 μM Aβ42. This sequence is identical in the three human isoforms (E2, E3, and E4) of apoE protein. Lysates from Aβ42-incubated synaptosomes were immunoprecipitated with immobilized anti-Aβ42 antibodies and the Aβ42-associated α7nAChR levels were determined by Western
blotting. Our ex vivo data as summarized in Fig. 1 indicate that ApoE<sub>133–149</sub> peptide added in vitro simultaneously or 10 min prior to Aβ<sub>42</sub> increased the abundance of Aβ<sub>42</sub>-α7nACHR complexes by 21.8 ± 6.4 to 39.7 ± 6.8% and 30.8 ± 7.4 to 45.4 ± 9.5%, respectively, with subtle dose dependency indicated by a 14.0 ± 1.8% increase by simultaneous addition of 0.05 μM apoE fragments with Aβ<sub>42</sub> (Fig. 1). Addition of apoE<sub>141–148</sub> in vitro simultaneously or 10 min prior to Aβ<sub>42</sub> increased the abundance of Aβ<sub>42</sub>-α7nACHR complexes by 21.9 ± 6.2 to 27.0 ± 5.6% and 18.7 ± 6.0 to 33.2 ± 10.3%, respectively, with slight dose-dependency as indicated by a 14.5 ± 4.4% increase by simultaneous addition of 0.05 μM apoE with Aβ<sub>42</sub> (Fig. 1). Substitution of lysine to leucine or aspartate residues in apoE133–149 and ApoE141–148, respectively, and scrambled apoE141–148 eliminated the effect of apoE<sub>133–149</sub> and apoE<sub>141–148</sub> on the Aβ<sub>42</sub>-α7nACHR interaction (Fig. 1a and b). In contrast, similar incubation of the rat FCX synaptosomes with apoE<sub>133–140</sub> did not alter the Aβ<sub>42</sub>-α7nACHR association (Fig. 1a and b). The comparable

![Fig. 1](https://example.com/image1.jpg) **Fig. 1** ApoE<sub>141–148</sub> mediates apoE-induced Aβ42-α7nACHR association enhancement ex vivo in rat brain synaptosomes. Rat frontal cortical synaptosomes were incubated with 0.1–100 μM apoE either 10 min prior to or simultaneously with 0.1 μM Aβ<sub>42</sub>. Synaptosomes were collected by centrifugation, solubilized, and immunoprecipitated with anti-Aβ<sub>42</sub>. The level of Aβ<sub>42</sub>-associated α7nACHRs in anti-Aβ<sub>42</sub> antibody immunoprecipitates was shown by Western blot detection of α7nACHR in anti-Aβ<sub>42</sub> antibody immunoprecipitates and quantified by densitometric scanning (a) and (b). Separately, rat cortical synaptosomes were incubated with 0.01, 0.05, 0.1, and 10 nM of apoE<sub>133–140</sub> or apoE<sub>141–148</sub> simultaneously with 0.1 μM Aβ<sub>42</sub>. The level of Aβ<sub>42</sub>-associated α7nACHRs in anti-Aβ<sub>42</sub> antibody immunoprecipitates was demonstrated by Western blot detection of α7nACHR and quantified by densitometric scanning (c).

*p < 0.01, compared to Aβ<sub>42</sub> alone by Newman-Keuls multiple comparisons (n = 5). α7nACHR α7-nicotinic acetylcholine receptor, Aβ amyloid beta, ApoE apolipoprotein E, IP immunoprecipitation.
β-actin levels in anti-Aβ42/actin immunoprecipitates demonstrated equal immunoprecipitation efficiencies and loading.

The effects of the apoE LDL receptor binding domain fragments on the Aβ42-α7nAChR interaction were verified using a cell-free assay system with biotinylated α7nAChRs trapped on a streptavidin-coated plate [4]. As in the ex vivo experiments described above, the ApoE fragments were added simultaneously with, or 10 min prior to, 20 nM FITC-conjugated Aβ42. The level of Aβ42-α7nAChR association was measured by the residual FITC signals. The data summarized in Fig. 2 indicate that 0.01–100 μM apoE133–149 added in vitro either simultaneously with or 10 min prior to Aβ42 increased the level of Aβ42-α7nAChR complexes by 13.8 ± 5.7 to 94.1 ± 17.2% and 13.9 ± 5.3 to 84.0 ± 16.7%, respectively (Fig. 2). Similarly, the addition of 0.01–100 μM apoE141–148 in vitro both simultaneously and 10 min prior to Aβ42 increased the abundance of Aβ42-α7nAChR complexes by 34.2 ± 7.6 to 105.8 ± 12.3% and 28.1 ± 6.1 to 90.0 ± 12.5%, respectively (Fig. 2). In contrast, substitution of lysine to leucine or aspartate residues in apoE133–149 and apoE141–148, respectively, and scrambled apoE141–148 had no effect on the Aβ42-α7nAChR interaction (Fig. 2). The addition of apoE133–140 also did not alter Aβ42-α7nAChR interaction.

Effects of apoE LDL receptor binding domain fragments on Aβ42-Aβ42 association

To assess the possibility that apoE increases the Aβ42-α7nAChR complex level by facilitating Aβ42 already bound to α7nAChR, we determined the effects of various apoE LDL receptor binding domain fragments on the Aβ42-Aβ42 interaction using an established cell-free system with biotinylated Aβ42 trapped on a streptavidin-coated plate [4]. The biotin-tagged Aβ42 trapped streptavidin-coated 96-well plate was incubated with 0.1–100 μM apoE fragments for 10 min prior to the addition of 20 nM FITC-conjugated Aβ42. The results shown in Fig. 3 indicate that all six apoE LDL receptor binding domain fragments at concentrations up to 100 μM have negligible effects on the Aβ42-Aβ42 complex formation. These data suggest that apoE promotes Aβ42-α7nAChR interaction directly but not by facilitating Aβ42 binding to Aβ42 already associated with the α7nAChRs.

ApoE4 increases the Aβ42-α7nAChR association

Because APOE ε4 is a prominent late-onset AD risk factor and all apoE subtypes contain the LDL receptor binding
domain, we assessed whether different apoE subtypes differentially modulate the Aβ42-α7nAChR association. We used both in vitro and ex vivo methods. In the in vitro experimental paradigm, the biotinylated α7nAChR trapped streptavidin-coated plate was incubated with 0.01–100 nM recombinant human apoE isoforms in the presence of FITC-conjugated Aβ42. ApoE4 at the test concentrations increased the Aβ42-α7nAChR association by 17.9 ± 2.1 to 60.2 ± 6.3% (Fig. 4a); apoE3 promoted a much weaker enhancement of the Aβ42-α7nAChR interaction at 10 nM that did not reach statistical significance (13.6 ± 7.9% increase; Fig. 4a). Next, rat FCX synaptosomes were incubated with 0.01 μM of recombinant human apoE subtypes in the presence of Aβ42. ApoE4 at 0.1–10 μM increased the abundance of Aβ42-α7nAChR complexes by 34.9 ± 5.4 to 72.6 ± 8.7%, whereas apoE2 and apoE3 were without significant effects (Fig. 4b and c). The comparable β-actin levels in anti-Aβ42/actin immunoprecipitates demonstrated equal immunoprecipitation efficiencies and loading. These data together indicate that apoE4 can enhance the formation of Aβ42-α7nAChR complexes.

Specific apoE LDL receptor binding domain fragments increases Aβ42-induced tau phosphorylation
Aβ42 (0.1 μM) increased pS202, pT231, and pT181 by 450–703% within 30 min in FCX synaptosomes (Fig. 5a and b). Because apoE LDL receptor binding domain fragments that contain apoE141–148 promote Aβ42-α7nAChR interaction, we assessed their effects on Aβ42-induced, α7nAChR-dependent tau phosphorylation. Just as apoE141–148 containing peptides (apoE133–149 and apoE141–148) increased the Aβ42-α7nAChR interaction, incubation of apoE133–149 or apoE141–148 enhanced Aβ42-induced tau phosphorylation at all three phosphoepitope levels with similar efficacy. Densitometric quantification reveals that apoE133–149 and apoE141–148 increased Aβ42-induced pS202, pT231, and pT181 by 19.9 ± 5.7 to 58.3 ± 9.3% and 26.3 ± 7.1 to 40.8 ± 9.4%, respectively (Fig. 5a and b). Again, apoE133–149 with lysine to aspartate substitution, as well as scrambled apoE141–148 had no effect on Aβ42-induced tau phosphorylation (Fig. 5a and b). Similar incubation of the rat FCX synaptosomes with apoE133–149 did not have appreciable effects on Aβ42-induced tau phosphorylation at all three tau phosphoepitopes (Fig. 5a and b). The dose-dependency of apoE133–149 and apoE141–148 in promoting Aβ42-induced tau phosphorylation was further tested by simultaneous addition of 0.01–10 μM apoE133–149 and apoE141–148 with Aβ42. Similar to their effects on the Aβ42-α7nAChR association, apoE133–149 and apoE141–148 significantly increased Aβ42-induced tau phosphorylation on Serine202, Threonine231, and Threonine181 by 20.5 ± 5.3 to 54.9 ± 13.0% and 29.5 ± 6.7 to 62.3 ± 10.2%, respectively, starting at 0.05 μM (Fig. 5c). These data together confirm that the apoE4 isoform can promote Aβ-induced neurofibrillary lesions via the apoE141–148 region.
Increased Aβ42-α7nAChR association by plasma from patients with dementia due to AD and MCI subjects

The parallel increases in Aβ42-α7nAChR complex formation and Aβ42-induced tau phosphorylation by the fragments containing apoE141–148 suggest that apoE4 can facilitate AD pathogenesis by promoting the Aβ42-α7nAChR interaction. Previously, we have shown in synaptosomes derived from rodent and human postmortem brains that incubation of synaptosomes with exogenous Aβ42 promotes the formation of Aβ42-α7nAChR complexes to the levels of AD [4, 5, 7]. Using an ex vivo system, we determined the magnitude of the increase in the Aβ42-α7nAChR association induced by incubating rat FCX synaptosomes simultaneously with 0.1 μM Aβ42 and 25 μl plasma from patients of the ROSAS

Fig. 5 ApoE4 mediates ApoE-induced Aβ42-elicited α7nAChR-dependent tau phosphorylation. Rat frontal cortical synaptosomes were incubated simultaneously with 0.1 μM apoE fragments and 0.1 μM Aβ42. Synaptosomes were collected by centrifugation, solubilized, and immunoprecipitated with anti-tau antibodies. The levels of Aβ42-induced tau phosphorylation on the serine 202 (pS202tau), threonine181 (pT181tau), and threonine231 (pT231tau) in anti-tau immunoprecipitates shown were determined using Western blot detection of each phosphoepitope and quantified by densitometric scanning. Separately, rat cortical synaptosomes were incubated with 0.01, 0.05, 0.1, 1, and 10 nM apoE133–149 or apoE141–148 simultaneously with 0.1 μM Aβ42. The level of Aβ42-induced pS202tau, pT181tau, and pT231tau in anti-tau immunoprecipitates were determined by Western blot detection of each phosphoepitope and quantified by densitometric scanning. *p < 0.01, **p < 0.05, compared to Aβ42 alone by Newman-Keuls multiple comparisons (n = 4–6). Aβ amyloid beta, ApoE apolipoprotein E, IP immunoprecipitation
cohort with diverse APOE genotypes. Our data show that the $\beta_{42}$-α7nAChR complexes were more abundant when incubated with plasma from subjects with MCI (increased by 44.7 ± 6.7%) and AD (increased by 99.5 ± 3.6%) compared to plasma from controls (increased by 13.5 ± 4.1%) regardless of APOE genotypes in visit 1 (Fig. 6a and b). There were no discernible differences between visit 1 and the follow-up visit 2 (Fig. 6a and b). Using the percentage increase by the addition of plasma, our data indicate that apoE4 promotes the $\beta_{42}$-α7nAChR association: the levels of $\beta_{42}$-α7nAChR complexes progressively increased as the number of APOE ε4 alleles increased in MCI and AD cases (Fig. 6a and c). A significant correlation was found between the percentage increase by the addition of plasma and total MMSE score with an overall Spearman correlation coefficient of −0.71 (Fig. 6d). A significant correlation was also noted between the percentage increase and disease progression (reduction of the MMSE score) with an overall Spearman correlation coefficient of −0.57 (Fig. 6e). This finding is in contrast with APOE ε2 and APOE ε3 carriers, whose $\beta_{42}$-α7nAChR complex levels virtually held steady with fewer incidences of cognitive decline. Together, these data support the notion that apoE4 promotes AD pathogenesis by promoting $\beta_{42}$-α7nAChR complex formation.

$\beta_{42}$-α7nAChR complex levels and reduced response to exogenous $\beta_{42}$ in MCI and AD lymphocytes correlated with plasma apoE4 level

Because lymphocytes contain α7nAChRs and abundant CHRFAM7A and the $\beta_{42}$-α7nAChR complexes are more abundant in AD [7, 35], we assessed whether the $\beta_{42}$-α7-like nAChR complex levels are higher in the membranes of lymphocytes from AD and MCI patients and whether the abundance of $\beta_{42}$-α7-like nAChR complexes correlate with the APOE genotypes, especially the APOE ε4. We isolated lymphocytes from the buffy coat of a large cohort consisting of well-matched control-MCI-AD triads with diverse APOE genotypes at two time points. We determined the levels of $\beta_{42}$-α7-like nAChR complexes following ex vivo exposure to either 100 or 500 μM $\beta_{42}$. As reported previously [7], $\beta_{42}$-α7-like nAChR complex levels increased following exposure to exogenous $\beta_{42}$. Exogenous $\beta_{42}$ increased $\beta_{42}$-α7-like nAChR complex levels by 143.7 ± 14.8% in controls and by 91.9 ± 13.9% in MCI subjects, but by only 9.4 ± 1.0% in AD patients at visit 1 (Fig. 7a and b). This $\beta_{42}$-induced response did not change significantly in lymphocytes obtained at visit 2 (Fig. 7a and b). Corroborating plasma effects in rat cortical synaptosomes, the levels of $\beta_{42}$-α7-like nAChR complexes progressively increased along with increasing number of APOE ε4 alleles in the MCI and AD cases, as indicated by the reduced effects of exogenously added $\beta_{42}$ (Fig. 7a and c). The $\beta_{42}$-induced increases in $\beta_{42}$-α7-like nAChR complex levels in lymphocytes were significantly correlated with plasma-elicited increases in $\beta_{42}$-evoked $\beta_{42}$-α7nAChR association when segregated by diagnosis (Fig. 7d). As in rodent synaptosome experiments, a significant correlation was found between the $+\beta_{42}$-/−$\beta_{42}$ ratios in lymphocytes and the magnitude of decrease in MMSE scores with an overall Spearman correlation coefficient of 0.46 (Fig. 7e). There were, however, no discernible APOE ε genotype- or diagnosis-related changes in α7nAChRs and α7nAChR-like, CHRFAM7A protein levels in lymphocytes in this study cohort (Fig. 8a and b). Our data also show that α7nAChR and CHRFAM7A α7-like nAChR complexes with each other in the membranes of lymphocytes as indicated by the coimmunoprecipitation of α7nAChR and CHRFAM7A. However, there were no detectable APOE ε genotype-related changes in the α7nAChR/CHRFAM7A complex levels (Fig 8c and d).

Discussion

The present study shows that apoE4 interacts with α7nAChRs via the apoE LDL receptor binding domain, apoE1–apoE14, to increase $\beta_{42}$-α7nAChR association and $\beta_{42}$-elicited, α7nAChR-dependent tau phosphorylation. Plasma from APOE ε4 carriers increased $\beta_{42}$-α7nAChR complex levels in rat synaptosomes. The relevance of these in vitro and ex vivo results to AD pathogenesis is supported by higher abundance of $\beta_{42}$-α7-like nAChR complexes in AD and MCI lymphocytes, correlating with the APOE ε4 genotype in hetero- and homozygous APOE ε4 carriers. Underscoring the more rapid cognitive decline in APOE ε4 carriers, we present a novel mechanism through which apoE4 may facilitate the $\beta_{42}$-driven AD pathogenesis in both brain and peripheral cells. Conspicuously, plasma from all AD subjects (independent of APOE ε4 status) has a greater effect on promoting the $\beta_{42}$-α7nAChR association, and lymphocytes of AD subjects have more abundant $\beta_{42}$-α7-like nAChR complexes. These findings suggest that other factor(s) in addition to APOE ε4 may be present in AD. Neurotoxic apoE proteolytic products can be formed by neurons in APOE ε4 transgenic mice and in the brains and cerebrospinal fluid from AD patients, with the highest level found in APOE ε4 carriers [11, 27, 36–38]. Some synthetic apoE fragments are neurotoxic [12, 13]. Since the neurotoxic apoE fragments retain the LDL binding domain [36, 39], the increased $\beta_{42}$-α7nAChR interaction in AD may result from higher apoE toxic fragments that presumably increase with duration of disease, although their presence in the plasma of AD subjects is currently not known.

APOE ε4 accelerates the onset of both familial and late-onset sporadic AD with greater deleterious cognition...
Enhanced Aβ-α7nAChR association by plasma from APOEε4 carriers with MCI or dementia due to AD correlates with longitudinal cognitive decline. Rat frontal cortical synaptosomes were incubated simultaneously with 25 μl plasma and 0.1 μM Aβ42. The levels of Aβ42-α7nAChR complexes were determined by the abundance of α7nAChRs in the anti-Aβ42 antibody immunoprecipitates by Western blotting (a), quantified by densitometric scanning, and normalized by β-actin immunoreactivity as the immunoprecipitation/loading controls. The data expressed as the ratios of positive plasma to negative plasma (mean ± SEM) summarizes the effects of plasma derived from two separate visits on Aβ42-elicited the Aβ42-α7nAChR association in different diagnostic groups without (b) and with (c) segregating by the APOE genotype. *p < 0.01, **p < 0.05, compared to respective cognitive normal group or APOE ε2/ε3 by Dunnett’s test adjusted for multiple comparisons. 

d Correlation to baseline cognitive status defined by Mini-Mental State Examination (MMSE) score (n = 86): spearman correlation coefficient, controls = 0.19 (–0.23; 0.55); MCI = –0.32 (–0.61; 0.06); AD = –0.14 (–0.46; 0.22); all = –0.71 (–0.80; –0.59), e Correlation to longitudinal cognitive changes per evolution of diagnostic group (control not progressed (CTRL NP), MCI NP, and progressed (P), MCI NP, and P, and AD): spearman correlation coefficient controls NP = –0.19 (–0.58; 0.26); controls P = NA; MCI NP = –0.19 (–0.67; 0.40); MCI P = –0.22 (–0.64; 0.31); AD = –0.30 (–0.58; 0.05); all = –0.57 (–0.70; –0.41). α7nAChR α7-nicotinic acetylcholine receptor, Aβ amyloid beta, AD Alzheimer’s disease, apoE apolipoprotein, C controls, MCI mild cognitive impairment, V visit
Higher $\beta_42^{-\alpha_7nAChR}$ complex levels and reduced response to exogenous $\beta_42$ in lymphocytes from MCI and AD patients correlate with plasma apoE. Lymphocytes obtained from cognitive normal controls (C), subjects with mild cognitive impairments (MCI), and Alzheimer’s disease (AD) were incubated without or with 0.1 $\mu$M $\beta_42$. The levels of $\beta_42^{-\alpha_7nAChR}$ complexes were determined by the abundance of $\alpha_7nAChRs$ in the anti-$\beta_42$ antibody immunoprecipitates by Western blotting (a), quantified by densitometric scanning, and normalized by $\beta$-actin immunoreactivity as the immunoprecipitation/loading controls. The data expressed as the ratios of positive $\beta_42$ to negative $\beta_42$ (mean ± SEM) summarizes the effects of $\beta_42$ derived from two separate visits on the $\beta_42^{-\alpha_7nAChR}$ association in different diagnostic groups without (b) and with (c) segregating by the APOE genotype. *$p < 0.01$, **$p < 0.05$, compared to respective cognitive normal group b or APOE $\varepsilon2/\varepsilon3$ c by Dunnett’s test adjusted for multiple comparisons. d Correlations between positive plasma to negative plasma ratios in synaptosomes and positive $\beta_42$ to negative $\beta_42$ ratios in lymphocytes derived from visit 1 spearman correlation coefficient: controls = 0.17 (–0.25; 0.54); MCI = –0.81 (–0.91; –0.62); AD = –0.58 (–0.77; –0.30); all = –0.84 (–0.89; –0.76). e Correlation to longitudinal cognitive changes per evolution of diagnostic group (control not progressed (CTRL NP) and progressed (P), MCI NP and P, and AD), n = 86 including 32 AD, 30 MCI, and 24 control subjects from four distinct APOE genotype groups: controls NP = –0.04 (0.46; 0.40); controls P = NA; MCI NP = –0.08 (–0.61; 0.49); MCI P = –0.10 (–0.57; 0.42); AD = 0.23 (–0.12; 0.53); all = 0.46 (0.28; –0.62). $\alpha_7nAChR$ $\alpha_7$-nicotinic acetylcholine receptor, $\beta$ amyloid beta, apoE apolipoprotein, $V$ visit.
effects and neurodegeneration in women than in men [40–45]. APOE ε4 is associated with worse clinical outcome in traumatic brain injury [46], multiple sclerosis [47], Parkinson’s disease [48], frontotemporal dementia [49], and stroke [50]. ApoE fragments increase NFT-like intraneuronal inclusions in cultured neurons [27]. Peptide fragments derived from the apoE LDL receptor-binding domain interact with, and inhibit, α7nAChR [28–31]. However, these data do not directly support the known apoE4 role in promoting AD pathogenesis, even though α7nAChR is a receptor for Aβ and contributes to Aβ42-mediated AD pathologies [4–7, 32–33]. Our data showing that apoE4 promotes the Aβ42-α7nAChR association provides an essential link to AD pathogenesis. This hypothesis is supported by the AD-like neurodegeneration and behavioral deficits in transgenic mice expressing carboxy-terminal truncated apoE4 [34]. Although the apoE LDL receptor-binding domain is common to all apoE subtypes, recombinant humanapoE4 preferentially increases the Aβ42-α7nAChR association. This finding suggests that the conformational form of apoE, but not apoE3 or apoE2, exposes the apoE LDL receptor binding domain to α7nAChRs since the amino acid sequences of apoE subtypes are almost virtually identical. This hypothesis is supported by an earlier report that suggests that apoE4 is structurally different from apoE3 based on differences in hydrogen-deuterium exchange and site-directed mutations [51].

ApoE appears to regulate Aβ aggregation and deposition. Deletion of the APOE gene dramatically reduces fibrillar Aβ deposits in an AD transgenic mouse model [52] as well as apoE immunoreactivity in amyloid plaques in human AD brains [53]. By increasing the Aβ42-α7nAChR association, apoE4 can promote internalization of the Aβ42-α7nAChR complexes to facilitate formation of intraneuronal Aβ aggregates and amyloid plaques [2]. The elevated intraneuronal Aβ oligomers can impair intraneuronal mitochondria and lysosomes to drive neurodegeneration [18]. In agreement, Aβ-rich amyloid plaques are more abundant and commonly found in APOE ε4 carriers and AD patients with positive amyloid scans [14, 54–56]. Increased Aβ42-α7nAChR interaction by apoE4 suggests that amyloid plaques may form earlier and more readily in APOE ε4 carriers [57, 58]. Indeed, fibrillar Aβ deposits, the hallmark of AD and revealed by florbetapir (PiB) imaging, are more abundant and detected earlier in AD and even in cognitively normal APOE ε4 carriers versus noncarriers [57, 59]. Cognitively normal APOE ε4 carriers with positive amyloid imaging decline cognitively much earlier than noncarriers [59]. Compared to APOE ε4, APOE ε2 appears to associate with cognitive intactness in >90-year-old individuals even though APOE ε2 is also linked to higher amyloid plaque loads [60]. This reported APOE ε2 association with amyloid plaque levels is, however, not supported by
our finding that recombinant human apoE2 minimally alters $\alpha_7nAChR$ interaction (Fig. 4).

APOE e4 is also linked to the magnitude of neurofibillary lesions. Although apoE is primarily produced by astrocytes and microglia in healthy states, stress or injury induce neuronal apoE expression and produce neurotoxic apoE4 fragments to increase tau hyperphosphorylation, cytoskeletal disruption, and mitochondrial dysfunction, and eventual neurodegeneration [9, 37, 61, 62]. The notion that APOE e4 confers vulnerability to stress and injuries is supported by data demonstrating that neurons in APOE e4 carriers with temporal lobe epilepsy are more susceptible to seizure damage and to $A_\beta$ toxicities than those harboring APOE e3. [63]. Despite all these linkages, the mechanism responsible for apoE4-induced tau hyperphosphorylation remains unclear. Our earlier reports showed that either incubation of synaptosomes with $A_\beta_{42}$ or intraventricularly administered $A_\beta_{42}$ induced robust tau phosphorylation at three proline-directed cell types [64].

The dose-dependency in the apoE141–148 enhancement of $A_\beta_{42}$-induced tau phosphorylation, and that NFTs are related to $A_\beta_{42}$ internalization, deposition, and plaque formation [4, 5, 7]. As illustrated here, apoE4 can promote the $A_\beta_{42}$-$\alpha_7nAChR$ interaction via apolipoprotein E42-141–148 enhancement of $A_\beta_{42}$-induced tau hyperphosphorylation to exacerbate $A_\beta_{42}$-induced tau hyperphosphorylation that presumably leads to more extensive neurofibrillary lesion.

The differential effects of astrocyte-derived versus neuron-derived apoE4 on excito-toxic damage (the former protecting against and the latter enhancing) indicate that very different apoE proteolytic pathways exist in these two cell types [64].

The $\alpha_7nAChR$s in lymphocytes regulate the development and activation of these cells [65–67]. However, the $\alpha_7nAChR$ expression in lymphocytes from AD subjects either increased [68] or did not change [69] compared to their neurologically normal peers. Similarly, we did not find APOE genotype- or AD-related changes in $\alpha_7nAChR$-like protein levels in lymphocytes (Fig. 8). These studies suggest that changes in $\alpha_7nAChR$ and CHRFAM7A expression are likely unrelated to the increased pathogenic $A_\beta_{42}$-$\alpha_7$-like nAChR interaction in lymphocytes from AD subjects. The fact that markedly elevated $A_\beta_{42}$-$\alpha_7nAChR$ complexes in the brain parallels the increased $A_\beta_{42}$-$\alpha_7$-like nAChR association in lymphocytes of AD patients suggests that this association in lymphocytes could potentially serve as a noninvasive, blood-based AD diagnostic biomarker [4, 7].

A heightened $A_\beta_{42}$-$\alpha_7$-like nAChR interaction in lymphocytes is also observed in this cohort of AD subjects. The magnitude of the increase in the $A_\beta_{42}$-$\alpha_7$-like nAChR association in lymphocytes is significantly greater in APOE e4 carriers than with other APOE genotypes, even in AD cases. ApoE4 and perhaps neurotoxic apoE(4) fragments originating from neurons likely intensify the $A_\beta_{42}$-$\alpha_7$-like nAChR interaction to promote $A_\beta_{42}$-mediated AD pathogenesis. $A_\beta_{42}$-$\alpha_7$-like nAChR complex levels correlate with the rate of cognitive decline in the APOE e4 carriers (Fig. 6c), and current data suggest that enhancing the $A_\beta_{42}$-$\alpha_7$-like nAChR interaction may contribute to apoE4-induced AD pathologies. Hence, the $A_\beta_{42}$-$\alpha_7$-like nAChR complex level in lymphocytes may serve as a peripheral AD biomarker to indicate the presence of more extensive AD pathologies. Unlike the recent report using a plasma lipid profile to identify an early AD degenerative trait [70], blood samples in this study were only obtained from two time points. Future experiments will elucidate timeframes, particularly including presymptomatic time points, are needed to assess the utility of $A_\beta_{42}$-$\alpha_7nAChR$ complex levels in lymphocytes as a biomarker for AD dementia.

In addition to $\alpha_7nAChRs$, expression of the $\alpha_7nAChR$ chimeric gene, CHRFAM7A, was also found in the lymphocytes of humans [35]. CHRFAM7A functions as a negative regulator of $\alpha_7nAChRs$ in a coexpression study [35] and retains the binding site for $A_\beta$ [5, 32], although it is unclear whether $A_\beta$ binds to CHRFAM7A with similarly high affinity as for the $\alpha_7nAChRs$. Our data show that the expression levels of $\alpha_7nAChRs$ and CHRFAM7A in lymphocytes are similar in three diagnostic groups regardless of APOE genotype. Further, we found CHRFAM7A forms complexes with $\alpha_7nAChR$ in vivo in the membranes of lymphocytes, although the levels of $\alpha_7nAChR/CHRFAM7A$ complexes are comparable in different APOE genotypes and diagnostic groups. Importantly, the increased $A_\beta_{42}$ association with $\alpha_7nAChRs$ and/or CHRFAM7As in lymphocytes from AD subjects agrees with previous findings in postmortem human brains and in human lymphocytes [4, 7, 32].

The immune system interacts with the brain bidirectionally through common receptors and ligands, such as interleukin-1$\beta$ and other proinflammatory cytokines [71, 72]. We showed that the induction of plasticity-related phenomena in the brain similarly affects lymphocyte function [73]. Moreover, lymphocytes from senescent mice transferred to young mice decreased the learning abilities of these mice to the level of senescent mice and produced senescence-like serum-brain reactivity [74]. As in postmortem brains, lymphocytes derived from AD patients and ex vivo incubation of lymphocytes from normal controls with $A_\beta_{42}$ showed substantially higher $\alpha_7nAChR$-TLR4-filamin A complexes [7]. Our finding that $A_\beta_{42}$-$\alpha_7$-like nAChR complexes in lymphocytes
correlate with effects on the synaptic Aβ13-α7nAChR interaction by plasma from APOE ε4 carriers and AD patients suggests similar apoE4 influences in the brain and the periphery. We therefore believe that the Aβ13-α7-like nAChR complex level in lymphocytes may be used as an antecedent biomarker to gauge AD neuropathogenic progression during the prodromal phase of the disease given that pathological changes occur considerably earlier than cognitive impairments. This novel potential biomarker holds a higher pathogenic rationale than many other blood-based biomarkers such as lipid profiling [70] and autoantibody panels [75]. Neuroinflammation is intimately involved in AD, and certain systemic leukocytes are relatively long lived; it is then possible these immune cells detect neuronal pathological changes and respond by altering molecules within themselves such as T-cell activation markers or their phenotypes [76]. Together with our current finding of AD-related changes in lymphocytes, these data suggest that, during AD progression, brain pathologies may lead to systemic and long-term immunological changes in lymphocytes and other blood cells. Changes induced by apoE4 in peripheral immune cells such as increased Aβ42-α7nAChR interaction may be potential AD biomarkers.

Finally, apoE is required for deposition of Aβ fibrils in amyloid mouse models [52]. Genetic knockdown of human apoE reduces amyloid plaque loads in transgenic AD mouse models, regardless of apoE isofom [77]. Interestingly, Aβ12-28, which prevents the Aβ-α7nAChR interaction [4, 32], also blocks apoE-driven Aβ deposition and ameliorates memory deficits in AD transgenic mouse models with elevated amyloid [78]. Agents that reduce Aβ42-α7nAChR complex levels decrease Aβ aggregates, hyperphosphorylated tau (NFT), and synaptic pathology in AD mouse models [5-7, 79]. Hence, apoE4 promotes the Aβ42-α7nAChR interaction, blocking this interaction may prevent apoE4 and its toxic fragments from promoting Aβ-mediated α7nAChR-dependent AD pathogenesis in APOE ε4 carriers.

A few limitations warrant caution in drawing conclusions from this study. First, because clinical diagnosis is based mainly on cognitive symptoms, the precise brain AD pathologies are not known. Second, despite well-matched pairs, the number of cases in this study is small, especially in the APOE ε2/ε3 cohort. Third, the apoE peptides were used primarily to illustrate the phenomenon rather than to provide quantitative measurements. Last, although the increased Aβ42-α7nAChR complex levels correlate with progression of cognitive decline in AD, whether the Aβ42-α7nAChR association enhancement by apoE accelerates AD pathology is ambiguous. Further research is needed to fully elucidate the contribution of the apoE4-induced increase in the Aβ42-α7nAChR interaction to AD pathogenesis.

Conclusion

Our data obtained from well-matched pairs in the ROSAS cohorts suggests that increased lymphocyte Aβ42-α7nAChR-like complexes may be a potential biomarker for AD pathologies. Importantly, we show that apoE4 enhances the Aβ42-α7nAChR interaction through apoE141–148 to contribute to apoE4-driven, Aβ-mediated neurodyfunction and pathologies. These unique agents that prevent or disrupt the Aβ42-α7nAChR association should be considered as disease-modifying therapeutics for AD patients, including APOE ε4 carriers.

Abbreviations

α7nAChR: α7-Nicotinic acetylcholine receptor; Aβ: Amyloid beta; AD: Alzheimer’s disease; ApoE: Apolipoprotein E; CDR: Clinical Dementia Rating; DMSO: Dimethyl sulfoxide; DSM-IV: Diagnostic and Statistical Manual of Mental Disorders, version IV; E2: Ethylenediaminetetraacetic acid; FOX: Frontal cortex; FITC: Fluorescein isothiocyanate; K-R: Krebs-Ringer; LDL: Low-density lipoprotein; MCI: Mild cognitive impairment; MMSE: Mini-Mental Status Examination; NFT: Neurofibrillary tangle; PAGE: Polyacrylamide gel electrophoresis; PBS: Phosphate-buffered saline; SDS: Sodium dodecyl sulfate; Tris: 2-Amino-2-(hydroxymethyl)propane-1,3-diol

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Availability of data and materials

All data generated or analyzed during this study are reported in this article. The raw datasets of the human lymphocytes in the current study are not publicly available due to commercial interests of the Institut De Recherche SERVIER but are available from the corresponding author on reasonable request.

Authors’ contributions

H-YW designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. CT-T designed, provided guidance to the selection of lymphocyte samples and experimental design, and edited the manuscript. AS, SMS, JK, and AK performed tissue preparation, and in vitro and ex vivo experiments, as well as helped in experimental designs and manuscript preparations. PM was a major contributor of in vitro and ex vivo experimental design. IG, EB, and KD managed clinical data collection and analysis and edited the manuscript. EM provided guidance for experimental and clinical design as well as manuscript preparation. P-JO and BV conducted all clinical assessments, sample collections and provided clinical study design. MP and VK oversaw the clinical study design and progression, provided clinical data analysis, and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures comply with the National Institutes of Health Guide for Care Use of Laboratory Animals and were approved by the City College of New York Animal Care and Use Committee (IACUC) Protocol no. 836.1. Human participants and their informed caregiver took part in the study on a voluntary basis, and they gave their written informed consent at selection. The ethics committee of Toulouse University Hospital approved the study protocol and all its amendments (registration number DGS 20060500).

Consent for publication

Not applicable.
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