Stabilization of intracellular trafficking and metabolism of amyloid β-protein precursor and Alcadeinβ by Apolipoprotein E

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Running head: Intracellular metabolism of APP and Alcadeins by ApoE
Abstract
Intracellular metabolism of amyloid β-protein precursor (APP) is important for the pathogenesis of Alzheimer’s disease (AD). Alcadeins (Alcα, Alcβ, and Alcγ) are neural membrane proteins similar to APP in their localization, metabolism, and cellular function. Isoform ε4 of apolipoprotein E (ApoE) is major risk factor for AD. We found that ApoE expression attenuated intracellular trafficking of APP and Alcβ, resulting in metabolic stabilization of both proteins. By contrast, Alcα intracellular proteolysis was facilitated by ApoE expression, which was not due to an increase in the primary cleavage of Alcα. This difference may result from binding of ApoE to membrane proteins.

Key words; APP, Alcadein, Apolipoprotein E, Alzheimer’s disease

Abbreviations. AD, Alzheimer's disease; Alcs, Alcadein family proteins; Alcα, Alcadein α; Alcβ, Alcadein β; Alcγ, Alcadein γ; APP, amyloid β-protein precursor; Aβ, β-amloid; p3-Alc, small peptide generated by α- and γ-site cleavages of Alc; sAlc, amino-terminal extracellular domain fragments of Alc secreted by a cleavage of Alc by primary secretase; sAPP, amino-terminal extracellular domain fragments of APP secreted by cleavage of APP by primary secretases.
Bullet point

ApoE stabilizes intracellular trafficking and metabolism of APP and Aβ.
1. Introduction

Alzheimer’s amyloid β-protein precursor (APP) is a type I membrane protein from which amyloid-β protein (Aβ) is generated by serial proteolytic cleavages [1, 2]. The alcadeins (Alcs) are a family of evolutionarily conserved neural type I membrane proteins, Alcα, Alcβ, and Alcγ, being Alcα and Alcβ highly expressed in adult neurons. Alcα was isolated as a binding partner of neural adaptor protein X11-like (X11L), and is therefore also called XB31 (X11L-binding protein clone number 31); they were also characterized as postsynaptic calcium-binding proteins, calsyntenins [3, 4]. Because X11L itself was identified as an APP binding partner, Alc and APP are prone to form a tripartite complex mediated by cytoplasmic X11L [3, 5, 6]. Therefore, Alc colocalizes extensively with APP in healthy neurons and also in dystrophic neurites in senile plaques of Alzheimer’s disease (AD) patients [3].

Both APP and Alcs are cleaved primarily at the juxtamembrane region by APP α-secretase (ADAM 10 and ADAM 17). These cleavages result in secretion of large N-terminal fragments, sAPPα and sAlcs, into the extracellular milieu, along with generation of the membrane-associated C-terminal fragments APP CTFα and Alc CTF [6, 7]. APP, but not Alc, is also subject to an alternative primary cleavage by β-secretase (BACE1) in endosomes to generate amyloidogenic APP CTFβ [8]. The APP CTFs and Alc CTF are further cleaved by γ-secretase, which generates p3 peptide from APP CTFα, Aβ from APP CTFβ, and p3-Alc peptide from Alc CTF [1, 7, 9]. Thus, it is conceivable that APP and Alcs are subjected to similar metabolic regulation in neurons [1, 10]. Furthermore, Alc is subject to intracellular transport by kinesin-1 in neurons, as is APP, indicating that both Alc and APP may share similar metabolic fates during intracellular transport [11-16].

While the role of APP is clear in AD [1, 2, 8], the contribution of Alc and its metabolic fragments in AD pathogenesis has not been fully analyzed. Because both APP and Alcα/calsyntenin-1 function as major anterograde vesicular cargos of kinesin-1, well-organized performance of both cargo-receptor proteins may be important for a persistence of neuronal functions, and the inappropriate interactions of Alcα- and APP-containing vesicles with kinesin-1 are thought to promote aberrant APP metabolism including the increased generation of Aβ [12, 17, 18]. Furthermore, Alcβ/calsyntenin-3 is reported to function in the synapse formation and/or function [19,
suggesting that Alcβ may involve in Aβ-induced neurotoxicity.

For sporadic AD, which is not associated with pathogenic mutations in any of three major causative genes, APP, presenilin1 (PS1), or presenilin2 (PS2), the predominant risk factor is apolipoprotein E isoform ε4 (ApoE4), a protein that may be involved in Aβ clearance [21, 22]. However, it remains unknown whether ApoE participates in the intracellular metabolism of APP and Alcs. In this study, we examined the effect of ApoE expression on intracellular trafficking and metabolism of APP and Alcs.

2. Materials and Methods

2.1. Plasmid construction

The human Alcα cDNAs, pcDNA3.1-Alcα and pcDNA3.1-Alcβ, and FLAG-tagged pcDNA3.1-FLAG-Alcα and pcDNA3.1-FLAG-Alcβ, were described previously [7]. Human APP695 cDNAs, pcDNA3.1-APP, and pcDNA3.1-FLAG-APP were described previously [23, 24]. Human ApoE4 cDNA was prepared from brain of the human ApoE4 knock-in mouse [25], and human ApoE2 and ApoE3 cDNAs were prepared from ApoE4 cDNA with megaprimers and Ex Taq polymerase (Takara Bio). These ApoE cDNAs were inserted into the pcDNA3.1 vector at EcoRI/XhoI sites to generate pcDNA3.1-ApoE4, pcDNA3.1-ApoE3 and pcDNA3.1-ApoE2. The same cDNAs were also inserted into pcDNA3.1-C-FLAG and pcDNA3.1-C-HA vectors to generate pcDNA3.1-ApoE-FLAG and pcDNA3.1-ApoE-HA, which express C-terminal tagged ApoE proteins in cell. The FLAG-tagged human Alcα HA mutant cDNA was prepared by PCR; specifically, the DNA sequence encoding nine amino acids, A-N-H-M-A-A-Q-P-Q, around the α-secretase cleavage sites was replaced with a DNA sequence encoding the HA-tag to yield pcDNA3.1-FLAG-Alcα HAmut.

2.2. Transfection of cells, Western blot analysis, and co-immunoprecipitation assay

Neuro-2a (N2a) mouse neuroblastoma cells (0.3–1.0 × 10⁶) were transiently transfected with the indicated amounts of plasmid using Lipofectamine 2000 (Invitrogen/Life Technologies). After transfection for 24–30 h, the medium was changed for further culture. To analyze cellular proteins, cells were harvested and lysed
in a radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail. In order to analyze secreted proteins, FLAG-tagged sAlcα, sAlcβ, and sAPP were recovered from conditioned medium by immunoprecipitation with an anti-FLAG antibody and Protein G–Sepharose. Cell lysates and the immunoprecipitates were analyzed by Western blotting with the indicated antibodies, detected by ECL (GE Healthcare), and quantitated using VersaDoc Model 3000 (Bio-Rad Laboratiries).

In co-immunoprecipitation assays, cells were lysed with CHAPS lysis buffer (10 mM CHAPS in PBS including a protease inhibitor cocktail). After centrifugation (15,000 × g for 10 min), the indicated antibodies and Protein G–Sepharose beads were added to the lysate supernatant and incubated for 8–12 h with agitation. The beads were recovered by centrifugation and washed with CHAPS lysis buffer, and the immunoprecipitates were analyzed by Western blotting using the indicated primary antibodies along with horseradish peroxidase (HRP)-linked anti-rabbit or anti-mouse IgG secondary antibody.

2.3. Cell-surface biotinylation
Cells were washed with ice-cold PBS and incubated with 0.5 mg/mL of NHS-LC-Biotin (Thermo Fisher Scientific) in PBS for 30 min at 4°C. The cells were further incubated with ice-cold 50 mM glycine in PBS for 5 min and washed with ice-cold PBS, and then subjected to lysis in RIPA buffer containing protease inhibitor cocktail. After centrifugation (15,000 × g for 10 min), NeutrAvidin agarose beads (Thermo Fisher Scientific) were added to the supernatant of the lysate and incubated for 2 h under rotation. The beads were recovered by centrifugation and washed with RIPA buffer, and the bound proteins were analyzed by Western blotting.

2.4. Antibodies
Monoclonal mouse anti-FLAG (M2, Sigma-Aldrich), anti-HA (12CA5, BD Biosciences) and anti-α-tubulin (DM1A, Santa Cruz Biotechnology) antibodies, and polyclonal rabbit anti-human ApoE (#18171, IBL), anti-APP C-terminal (#8717, Sigma-Aldrich) antibodies were purchased from the indicated suppliers. Polyclonal sheep anti-mouse IgG HRP-linked species-specific whole antibody (NA931) and donkey anti-rabbit IgG HRP-linked species-specific whole antibody (NA934) were purchased from GE Healthcare. Polyclonal rabbit anti-Alcadelin α (#83) and
anti-Alcdein β (#99) antibodies were described previously [6].

3. Results

3.1. Stabilization of intracellular metabolism of APP and Alcβ, but not Alcα.

To investigate the basic intracellular metabolism of APP and major Alc species, Alcα and Alcβ, under the expression of ApoE, we performed a pulse-chase study in the presence or absence of ApoE4 expression (Fig. 1). N2a cells expressing APP, Alcα, and Alcβ with or without ApoE4 were cultured in the presence of cycloheximide, and cells were harvested at the indicated times for analysis of intracellular protein metabolism by Western blotting. Consistent with a previous report [26], immature APP (imAPP) with N-glycosylation was subject to O-glycosylation during maturation in the late Golgi, and mature APP (mAPP) gradually disappeared as a result of cleavage by APP α- and/or β-secretases (Fig. 1A). The half-life of total APP (mAPP plus imAPP) metabolism was 42.7 ± 3.8 min. In the presence of ApoE4, mAPP metabolism was not changed (i.e., the half-lives of mAPP in the presence or absence of ApoE4 were almost identical), whereas the imAPP level was not reduced. The observation indicates that ApoE4 expression suppressed APP maturation, which means that ApoE4 may attenuate the intracellular transport of APP into the late secretory pathway; consequently, the intracellular level of imAPP did not decrease. Therefore, the half-life of total APP metabolism was significantly stabilized (to 75.0 ± 9.6 min) in the presence of ApoE4, regardless of the invariant rates of mAPP metabolism.

This tendency was also observed for Alcβ (Fig. 1C). The half-life of total Alcβ (both Alcβ with either complex or high-mannose N-glycans) was slightly extended (to 197.8 ± 37.3 min in the presence of ApoE4 from 136.4 ± 13.6 min in the absence of ApoE4, although this difference was not statistically significant at the time points studied. However, Alcβ with high-mannose N-glycan (hAlcβ) tended to exhibit a longer half-life and was statistically significant at 240 min in the presence of ApoE4, suggesting again that ApoE4 attenuates Alcβ intracellular transport by retaining hAlcβ for a longer time in the early secretory pathway, as in the case of imAPP. As for mAPP, the metabolism of Alcβ with complex N-glycan (cAlcβ) in the presence of ApoE was almost the same in the absence of ApoE.
In contrast to APP and Alcβ, the half-life of total Alcα was shortened to 44.3 ± 7.3 min in the presence of ApoE4 from 73.1 ± 14.0 in the absence of ApoE4 (Fig. 1B). This change in the total amount of Alcα (i.e., Alcα with either complex or high-mannose N-glycans) was statistically significant at 30 and 60 min, indicating that metabolism of Alcα was facilitated in the presence of ApoE4. cAlcα and hAlcα also showed a shorter half-life, significantly at 30 and/or 60 min. Overall, intracellular metabolism Alcα, in contrast to that of Alcβ and APP, was facilitated or at least not delayed by co-expression of ApoE4. ApoE4 levels also decreased gradually along with chase time because cellular ApoE was secreted.

ApoE4 is the isoform associated with the greatest risk of AD onset, and it differs from ApoE2 and ApoE3 in various functions including Aβ metabolism [27, 28]. To reveal the effect of ApoE isoforms on the intracellular behavior of APP and Alc, we examined ApoE isoform-specific effects. In these experiments, APP or Alc was expressed in N2a cells with ApoE ε2, ε3, or ε4 isoforms, and changes of intracellular Alc and APP levels were examined by Western blotting (Fig. 2). All ApoE isoforms decreased the intracellular Alcα level to the same extent statistically significantly, but had no effect on Alcβ and APP levels, indicating that the effect of ApoE on intracellular APP and Alc metabolism is isoform-independent. ApoE4 and ApoE3 protein expression levels were slightly lower than that of ApoE2, regardless of the almost equal levels of mRNA (Fig. 2B and C), possibly reflecting a difference in stability among ApoE isoforms in the cell. Hereafter, we used ApoE4 for further analysis.

3.2. Primary cleavage of APP and Alc is not altered by co-expression of ApoE

The results of the pulse-chase study suggested that ApoE expression does not alter the primary cleavages of APP and Alcs: the reductions in the levels of mAPP and cAlcs, which predominantly appear on the cell surface, were not remarkable in the presence of ApoE. However, in contrast to imAPP, which stays in the ER and early Golgi, hAlcs appeared at some level on the cell surface, where they were cleaved by α-secretase. Therefore, a reduction in intracellular Alcα level may be caused by elevated primary cleavage of Alcα, but not APP or Alcβ. We investigated whether ApoE co-expression promoted the primary cleavage of APP and/or Alcs. Following primary cleavage, the
amino-terminal regions of APP and Alcα are secreted as sAPP, sAlcα, and sAlcβ respectively, [7]. We investigated changes in primary cleavage in cells expressing APP, Alcα, or Alcβ, with or without co-expression of ApoE4 (Fig. 3). Medium of N2a cells expressing FLAG-tagged APP, Alcα, or Alcβ, together with or without ApoE4-HA, were subjected to immunoprecipitation with FLAG antibody and the immunoprecipitates were examined by Western blotting with the same antibody. Notably, production of all three amino-terminal regions (sAPP, sAlcα, and sAlcβ) was decreased when ApoE4 was co-expressed (Fig. 3A): generation of sAPP and sAlcβ decreased by ~80%, whereas sAlcα secretion decreased by ~50%. The reductions in APP and Alcβ cleavage may be due to the slight decrease in cell-surface APP and Alcβ (Fig. 3B), which resulted in metabolic stabilization by ApoE expression (Fig. 1).

In contrast to APP and Alcβ, the cleavage of Alcα significantly decreased (Fig. 3A), and the cell-surface level of Alcα clearly increased (Fig. 3B). This observation indicates that cell-surface cleavage of Alcα by α-secretase does not contribute to the reduced level of intracellular Alcα in cells expressing ApoE4.

To confirm this finding, we introduced amino-acid substitutions around the primary cleavage site (Fig. 4A). When the resulting mutant, Alcα (HAmut), containing the HA-tag sequence instead of the α-cleavage sequence, was expressed in N2a cells, Alcα HAmut was remarkably stable with respect to primary cleavage. Large amounts of Alcα HAmut, especially cAlcα, were detected relative to Alcα in wild-type (WT) in cells without ApoE4 expression (Fig. 4B, -). By contrast, the intracellular levels of Alcα HAmut and WT were decreased by 30% upon co-expression of ApoE4. These data strongly indicate that the reduction in the level of Alcα in cells expressing ApoE is not due to an increase in primary cleavage. Furthermore, except for the primary cleavage, Alcα was subject to more intracellular degradation in the presence of ApoE.

3.3. Interactions of ApoE4 with APP and Alc.

ApoE and the extracellular/luminal region of APP and Alcα may face the lumen of organelles and transport vesicles in the secretory pathway. Hence, we asked whether intracellular ApoE can bind Alc or APP.

N2a cells expressing APP, Alcα, or Alcβ with ApoE4-FLAG were lysed, and co-immunoprecipitation assay was performed using the FLAG antibody (Fig. 5). The antibody co-immunoprecipitated Alcβ with high-mannose N-glycan along with
FLAG-ApoE4 (Fig. 5C), whereas FLAG-ApoE4 did not co-precipitate Alcα (Fig. 5B). The data show that ApoE4 associates with Alcβ in the ER or early Golgi, but not in the late secretory pathway. ApoE4 bound neither hAlcα nor cAlcα, indicating that Alcα and ApoE do not engage in intracellular interaction.

We examined intracellular localization of APP, Alcα or Alcβ in the presence or absence of ApoE4. N2a cells were transiently expressed with FLAG-tagged APP, Alcα and Alcβ in the presence or absence of ApoE4-EGFP expression, and subjected to immunostaining analysis with anti-FLAG and anti-EGFP antibodies. APP and Alcβ were strongly colocalized with ApoE4 in perinuclear Golgi-like structure (white color in merged panels), and the signals of APP and Alcβ in cytoplasmic region decreased when compared to these in cells without ApoE4 expression (magenta color in merge panels), while the localization of Alcα were largely independent of ApoE4 staining in Golgi and detectable in the cytoplasmic region even in the presence of ApoE4 (Supplementary figure). These observations support that ApoE4 associates to APP and Alcβ, but not to Alcα, to untransport APP and Alcβ in perinuclear early secretory compartment.

The interaction of hAlcβ with ApoE is consistent with the stabilized intracellular metabolism of hAlcβ, in contrast to that of cAlcβ (Fig. 1). Alcβ may include the ApoE-binding domain, and the complex N-glycan of Alcβ may inhibit ApoE binding. ApoE4 weakly bound both mature and immature APP (Fig. 5A), consistent with reports that the Aβ sequence and amino-terminal ecto-domain of APP bind to ApoE [29, 30]. Thus, it is conceivable that among APP, Alcα, and Alcβ, only Alcα cannot bind ApoE intracellularly, and that proteolysis of Alcα is facilitated en route to the cell surface [31].

4. Discussion

In this study, we investigated the effect of ApoE expression on the intracellular metabolism of APP and Alcs. We made several key observations: (i) APP metabolism was slowed down in the presence of ApoE by attenuation of the intracellular trafficking of APP, resulting in slower maturation of APP in the late secretory pathway; Alcβ also exhibited a similar tendency. However, Alcα exhibited different intracellular metabolism when ApoE was co-expressed. ApoE did not metabolically stabilize Alcα via attenuation of Alcα intracellular trafficking, and the intracellular level of Alcα was reduced in the presence of ApoE. (ii) The effect of ApoE is not isoform-specific, and
may depend on the binding between type I membrane protein and ApoE. (iii) ApoE did not influence the primary cleavage of APP and Alcs, although the attenuated intracellular trafficking of APP and Alcβ decreased the secretion of the primary cleavage products. (iv) The intracellular level of Alcα decreased regardless of primary cleavage of Alcα, suggesting that intracellular proteolysis of Alcα was facilitated by co-expression of ApoE.

Although the results summarized above are not sufficient to understand the molecular mechanism underlying the effect of ApoE co-expression on the intracellular behavior of APP and Alcs, these results suggest that ApoE stabilizes the metabolism and intracellular trafficking of APP and Alcβ, probably by luminal binding of ApoE to APP and Alcβ. Furthermore, ApoE expression may facilitate Alcα intracellular metabolism. Notably, Alcα tends to be subject to constitutive proteolysis prior to cell-surface exposure [31]. In fact, the cell-surface level of Alcα is lower than those of APP and Alcβ, regardless of ApoE expression (Fig. 3B). ApoE expression might facilitate this constitutive proteolysis of Alcα via an unidentified regulatory mechanism.

ApoE is usually synthesized in glial cells, whereas the ApoE receptor is expressed in neurons [27, 32]. However, several studies have demonstrated neural expression of ApoE [33, 34] and the particular functions of neural ApoE in the neurodegenerative state have been reported [35-37]. Moreover, the differences in the intracellular metabolism of Alcα relative to that of APP and Alcβ in response to ApoE co-expression suggests a possible role for ApoE in an intracellular trafficking of membrane proteins. APP and Alcs are cargo receptors that associate with the kinesin-1 motor [12-14, 16, 17] and recruit various proteins into cargo vesicles. The interaction of ApoE with the luminal/extracellular domain of cargo-receptor may influence the cytoplasmic structure of cargo receptors to regulate intracellular trafficking of cargo vesicles. Although analysis of this mechanism will require investigation, the present findings regarding the metabolic stabilization of APP by ApoE provides an insight into the pathobiology of AD, including the regulation of Aβ generation.

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small peptide sequence is sufficient for initiating kinesin-1 activation through part of TPR region of KLC1. Traffic 13, 834-848.


235-238.


**Figure Legends**

**Figure 1. Pulse-chase analysis of Alcα, Alcβ, and APP: Effect of ApoE expression**

N2a cells were transiently transfected with APP (A), Alcα (B), or Alcβ (C) plasmid (0.3 µg) with or without ApoE4 plasmid (0.1 µg), and then cultured for 26 h. Cells were further cultured in a medium containing cycloheximide (0.1mg/ml) for the indicated chase time (min) and harvested. Cell lysates were analyzed by Western blotting with anti-Alcα, anti-Alcβ, anti-APP, anti-ApoE, and anti-α-tubulin antibodies. APP (A) with N-glycan alone (imAPP) or both N- and O-glycans (mAPP) was quantitated along with the total amount of APP (mAPP plus imAPP). Alcα (B) and Alcβ (C) with complex N-glycan (cAlc) or high-mannose N-glycan (hAlc) was quantitated along with the total amount of Alc (cAlc plus hAlc). Closed circles indicate metabolism of APP and Alc in cells without co-expression of ApoE4, and open squares indicate metabolism of APP and Alc in cells co-expressing ApoE4. The value at 0 min was defined as 1.0; values shown represent means ± S.E. Asterisks indicate statistical significance as determined by Student’s t-test (n = 3 in panel A and n=4 in panels B and C; *, p < 0.05; **, p<0.01).

**Figure 2. Effect of ApoE on the metabolism of APP and Alcs is isoform-independent.**

(A) N2a cells were transiently transfected with APP, Alcα, or Alcβ plasmid (0.3 µg) along with ApoE2, ApoE3, or ApoE4 plasmid (0.1 µg), and then cultured for 24 h. Minus (-) indicates empty vector alone. APP, Alcα, and Alcβ were detected by Western blotting and their expression levels were quantitated (n=5). The value in the absence (-) of ApoE expression was defined as 1.0; values shown represent means ± S.E. The asterisks indicate statistical significance as determined by Tukey’s multiple comparison test (*, p < 0.05; **, p < 0.01). n.s, not significant. (B) Protein expression levels of ApoE isoforms in panel A were quantitated. The value in the presence of ApoE2 expression was defined as 1.0; values shown represent means ± S.E. The asterisks indicate statistical significance as determined by Dunnett’s multiple comparison test (n = 5; **, p < 0.01; ***, p < 0.001). (C) mRNA expression levels of ApoE isoforms
were confirmed by RT-PCR, and the amplified products were quantitated along with the level of β-actin mRNA. The value in the presence of ApoE2 expression was defined as 1.0; values shown represent means ± S.E. (n = 5).

Figure 3. Primary cleavage and cell-surface levels of APP and Alcs in cells expressing ApoE.
(A) Effect of ApoE4 on primary cleavage of APP, Alcα, and Alcβ. N2a cells were transiently transfected with FLAG-tagged APP, Alcα, or Alcβ plasmids (0.3 µg) in the presence (+) or absence (-) of ApoE4-HA plasmid (0.1 µg). (upper panel) FLAG-sAPP, FLAG-sAlcα, and FLAG-sAlcβ secreted into the medium were recovered by immunoprecipitation with FLAG antibody, detected by Western blotting with the same antibody, or quantitated. (lower panel) The value in the absence (-) of ApoE4 expression was defined as 1.0; values shown represent means ± S.E. The asterisks indicate statistical significance as determined by Student’s t-test (n = 3; *, p < 0.05; **, p < 0.01). (B) Effect of ApoE4 on cell-surface localization of APP, Alcα, and Alcβ. N2a cells were transiently transfected with FLAG-tagged APP, Alcα, and Alcβ plasmids (0.3 µg) in the presence (+) or absence (-) of ApoE4-HA plasmid (0.1 µg). The cell-surface biotinylated proteins were recovered from cell lysates with NeutrAvidin beads. (Upper panel) Lysates (total) and biotinylated proteins (cell-surface) were analyzed by Western blotting with FLAG, ApoE, and α-tubulin antibodies. (Lower panel) Cell-surface levels of APP, Alcα, and Alcβ in the presence (+) or absence (-) of ApoE4 expression. Values in the absence of ApoE4 were defined as 1.0; values shown represent means ± S.E. The asterisks indicate statistical significance as determined by Student’s t-test (n = 4; *, p < 0.051).

Figure 4. Intracellular metabolism of primary cleavage–resistant Alcα mutant in cells with or without expression of ApoE4
(A) Schematic structure of Alcα and amino-acid sequence around a cleavage site of α-secretase. In the HA mutant (HAmut), the amino-acid sequence (red) of Alcα wild-type (WT) was replaced with the HA-tag sequence (green), which prevents Alcα from undergoing primary cleavage. (B) Effect of HA mutation on cellular metabolism of Alcα upon co-expression of ApoE4. N2a cells were transiently transfected with FLAG-tagged wild-type (WT) or HA-mutated (HAmut) Alcα plasmid (0.3 µg) in the
presence (+) or absence (-) of ApoE4 plasmid (0.1 µg). (Left panel) Cell lysates were analyzed by Western blotting with FLAG and ApoE antibodies, and Alcα with complex N-glycan (cAlcα) and high mannose N-glycan (hAlcα) were detected. (Right panel) Total amount of Alcα (cAlcα plus hAlcα) was quantitated. The values in the absence (-) of ApoE4 expression were defined as 1.0; values shown represent means ± S.E. The asterisks indicate statistical significance as determined by Student’s t-test (n = 3; ***, p< 0.001).

**Figure 5. Binding of ApoE to APP and Alcs.**
N2a cells were transiently transfected with (A) APP, (B) Alcα, or (C) Alcβ plasmid (0.6 µg) with (+) or without (-) ApoE4-FLAG plasmid (0.3 µg). Cell lysates were subject to co-immunoprecipitation with FLAG antibody, and immunoprecipitates and lysates were detected by Western blotting with APP, Alcα, and Alcβ antibodies along with FLAG and α-tubulin antibodies. mAPP, APP with N- and O-glycans; imAPP, APP with N-glycan alone; cAlc, Alc with complex N-glycan; hAlc, Alc with high-mannose N-glycan.
Fig. 1

(A) Incubate (min) 0 30 60 120 240

- APP
- APP + ApoE4

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(B) Incubate (min) 0 30 60 120 240

- Alcα
- Alcα + ApoE4

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(C) Incubate (min) 0 30 60 120 240

- Alcβ
- Alcβ + ApoE4

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**Fig. 2**

(A) Western blot analysis of APP, Alcα, and Alcβ in the absence or presence of ApoE or Alc. The relative ratios of APP, Alcα, and Alcβ to actin are shown for each condition (E2, E3, E4).

(B) Quantitation of ApoE and β-actin protein expression levels. The relative ratio of ApoE to β-actin is shown for each condition (E2, E3, E4).

(C) Quantitation of ApoE mRNA expression levels. The relative ratio of ApoE mRNA to β-actin mRNA is shown for each condition (E2, E3, E4).
(A) medium IP : M2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FLAG-APP</th>
<th>FLAG-Alcα</th>
<th>FLAG-Alcβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE4-HA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sAPP or sAlc</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

![Graph showing relative ratios](image1)

(B) FLAG-APP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FLAG-APP</th>
<th>FLAG-Alcα</th>
<th>FLAG-Alcβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell surface</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>total</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

![Graph showing relative ratios](image2)

Fig. 3
Fig. 4
Fig. 5