

Apolipoprotein Receptor 2 and X11 α/β Mediate Apolipoprotein E-Induced Endocytosis of Amyloid- β Precursor Protein and β -Secretase, Leading to Amyloid- β Production

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The homeostasis of amyloid- β (A β) in the brain is critical to the pathogenesis of Alzheimer's disease (AD). A β is a fragment of amyloid- β precursor protein (APP) generated in neurons by two proteases, β - and γ -secretases. APP and β -secretase, both present on cell surface, are endocytosed into endosomes to produce A β . The molecular mechanism by which neurons trigger the production of A β is poorly understood. We describe here evidence that the binding of lipid-carrying apolipoprotein E (ApoE) to receptor apolipoprotein E receptor 2 (ApoER2) triggers the endocytosis of APP, β -secretase, and ApoER2 in neuroblastoma cells, leading to the production of A β . This mechanism, mediated by adaptor protein X11 α or X11 β (X11 α/β), whose PTB (phosphotyrosine-binding) domain binds to APP and a newly recognized motif in the cytosolic domain of ApoER2. Isomorphic form ApoE4 triggers the production of more A β than by ApoE2 or ApoE3; thus, it may play a role in the genetic risk of ApoE4 for the sporadic AD. The mechanism, which functions independently from Reelin–ApoER2 interaction, also provides a link between lipid uptake and A β production, which may be important for the regulation of neuronal activity.

Key words: amyloid- β precursor protein; apolipoprotein E; amyloid- β ; β -secretase; endocytosis; Alzheimer's disease

Introduction

An excess brain level of amyloid- β (A β) is a major factor in the pathogenesis of Alzheimer's disease (AD) (Selkoe, 2001). A β is formed from proteolysis of amyloid- β precursor protein (APP) by β -secretase [BACE1 (β -site APP-cleaving enzyme 1); memapsin 2] (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Lin et al., 2000) and γ -secretase. APP and β -secretase, an aspartic protease with an optimal activity around pH 4 are both present on the cell surface, where at pH 7, β -secretase has only minimal activity (Lin et al., 2000). Both proteins are endocytosed into endosomes, a major cellular site for APP cleavage and A β production (Koo and Squazzo, 1994; Cook et al., 1997; Hartmann et al., 1997). β -Secretase is then recycled back to the cell surface (Huse et al., 2000; Walter et al., 2001). Therefore, β -secretase traffics between an active and a relatively inactive compartment to facilitate the production of A β . The

mechanism and regulation for the endocytosis of both proteins may influence cellular A β production and pathogenesis of AD.

Cellular trafficking of APP and β -secretase are mediated by interactions of motifs in their cytosolic domains with adaptor proteins. The endocytosis of APP is facilitated by the interaction of a YENPTY motif with several adaptor proteins including X11 α/β and Fe65 (for review, see King and Scott Turner, 2004), both of which are specifically present in the brain. β -Secretase has a "dileucine acid cluster" motif (He et al., 2002, 2003, 2005), which interacts with GGA proteins to facilitate the transport of the protease in the recycling but not the endocytic pathway (He et al., 2005; Wahle et al., 2005), although the two leucines in the motif are required for the latter (Huse et al., 2000; Pastorino et al., 2002). APP has also been shown to be a "carrier" for the endocytosis of the ectodomain of β -secretase, most likely by the interaction of the proteins in the assembly of endocytic vesicles on the cell surface (X. P. Huang et al., 2004).

Apolipoprotein E (ApoE) polymorphism is associated with the sporadic form of AD for which ApoE4, but not ApoE2 or ApoE3, is a risk factor (for review, see Huang, 2006). The molecular mechanism underlying this association is unclear. One of the possibilities is that ApoE4 supports a high level of A β production through a brain-specific receptor. This hypothesis led us to examine the influence of ApoE and the role of ApoE receptor 2 (ApoER2) in A β production in neuronal cells. ApoER2, a mem-

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ber of the low-density lipoprotein (LDL) receptor family, is specifically expressed in the brain. Its physiological function is to bind ApoE-containing lipoprotein particles to import cholesterol and lipids into brain cells. This process is essential for remodeling of neuronal membrane and mediating synaptic plasticity. Here we report that ApoE stimulated A β production through a coordinated endocytosis of ApoE, ApoER2, APP, and β -secretase. This mechanism involves adaptor protein X11 α/β , which facilitates the formation of a complex among ApoER2, X11, and APP. In this system, ApoE4 produces more A β than does ApoE2 or ApoE3.

Materials and Methods

cDNA vectors. Vectors for human β -secretase and Swedish mutant of APP were described previously (Lin et al., 2000). Vectors for human X11 α , X11 β , human LDL receptor-related protein (LRP), murine ApoER2, Reelin, and bovine ADAM-10 were kindly provided by Drs. Christopher C. J. Miller (Kings College London, London, UK), Ben Margolis (University of Michigan, Ann Arbor, MI), Guojun Bu (Washington University, St. Louis, MO), Johannes Nimpf (University of Vienna, Vienna, Austria), Tom Carrant (St. Jude Children's Research Hospital, Memphis, TN), and Falk Fahrenholz (Johannes Gutenberg University, Mainz, Germany), respectively. PCR was used for transferring murine ApoER2 gene into pcDNA6 with V5-His-Tag at the C terminus and for creating mutants of murine ApoER2 in pcDNA6. Phosphotyrosine-binding (PTB) domain of X11 α (residue 457–643) was amplified by PCR and inserted into pcDNA6. GST constructs of GST-PTB (457–643), GST–postsynaptic density-95/Discs large/zona occludens-1 (PDZ) (650–837) of X11 α , and GST constructs of ApoER2 cytosolic domain GST–ApoC1 (881–996), GST–ApoC2 (881–925), GST–ApoC3 (926–996), and GST–APPC (650–695) were amplified by PCR and inserted into plasmid pGEX6.1 (GE Healthcare, Uppsala, Sweden). All constructs were verified by sequencing. GST fusion proteins were produced in *Escherichia coli* BL21 strain and purified as described previously (He et al., 2003).

Antibodies. Goat polyclonal antibodies against recombinant pro- β -secretase were affinity-purified using Affigel (Bio-Rad, Hercules, CA)-immobilized recombinant β -secretase. Rabbit polyclonal APP antibody AB5228, AB5352, monoclonal APP antibody MAB1560, monoclonal Reelin antibody MAB5364, and control mouse IgG were obtained from Chemicon (Temecula, CA). Mouse monoclonal antibody for X11 α was purchased from BD Biosciences (San Jose, CA). Mouse monoclonal antibody for LRP was purchased from Calbiochem (La Jolla, CA). Monoclonal V5 antibody, hemagglutinin (HA)-tag antibody, Myc antibody, and polyclonal antibody for ApoER2 were purchased from Invitrogen (Carlsbad, CA). Rabbit polyclonal anti- β -actin was purchased from Novus Biologicals (Littleton, CO). Monoclonal apoE and apoE4 antibodies were purchased from MBL (Naka-ku Nagoya, Japan).

Lipoproteins. ApoE and ApoE4 were from Bidesign (Saco, ME); ApoE, ApoE2, ApoE3, and ApoE4 were from Alpha Diagnostic International (San Antonio, TX). Human plasma lipoprotein fractions were prepared as follows: out-dated plasma was obtained from the Oklahoma Blood Bank and was supplemented with antibiotics, preservatives, and phenylmethylsulfonyl fluoride to prevent oxidative and proteolytic damage (Manchekar et al., 2004). Very low-density lipoprotein (VLDL) [density (d) < 1.006 g/ml], LDL (d 1.006–1.063 g/ml), and high-density lipoprotein (HDL) (d 1.063–1.21 g/ml) were prepared by sequential ultracentrifugation and dialyzed against PBS. The contents of ApoE4 and total ApoE were analyzed for individual VLDL samples using Western blots with monoclonal antibodies. Quantity of bands was determined by optical scanning using STORM Scanner (GE Healthcare) and ImageQuant (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation and Western blots. Human embryonic kidney 293 (HEK293) cells were transfected as described previously (He et al., 2005) with the following combinations of plasmids: ApoER2/X11 α , ApoER2/X11 β , or ApoER2/X11 α /APPsw695. Cells were harvested 36 h after transfection and lysed on ice in lysate buffer [PBS, pH 7.4, with 1% NP-40 and 1% saponin with protease inhibitor mixture (Roche Diagnos-

tics, Indianapolis, IN)]. Cell lysates were cleared by centrifugation and incubated with indicated antibody for 4 h at 4°C. Immune complexes were isolated using protein G-agarose beads (Sigma, St. Louis, MO) and subjected to SDS-PAGE and Western blotting using specific antibodies as indicated.

Pull-down assays. For GST pull-down, GST fusion proteins were retrieved as described previously (He et al., 2003). The amount of protein recovered with glutathione beads was determined using Bio-Rad Protein Assay and diluted to yield a concentration of 1 mg of protein per milliliter of beads. Cell lysates from HEK293 cells transfected with X11 α or X11 β plasmids were cleared by centrifugation and aliquots of the supernatant were incubated with the same amount of GST fusion protein bound to glutathione beads in PBS buffer for 4 h at 4°C. For nickel-nitrilotriacetic acid (NI-NTA) pull-down experiments, lysates from HEK 293 cells over-expressed X11 α , X11 α /ApoER2, X11 β , or X11 β /ApoER2 were individually incubated with same amount NI-NTA beads (Qiagen, Valencia, CA) in PBS for 4 h at 4°C. The bound proteins on both types of pull-down beads were recovered with SDS sample buffer at boiling temperature and subjected to Western blot.

Cellular A β 40/A β 42 production. Neuroblastoma N2a-APPsw cells (from Dr. Riqiang Yan, Lerner Research Institute, Cleveland, OH) were cultured in 24-well plates in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen) with 80 μ g/ml G418 1 d before use. Transient transfections were done using FuGENE6 (Roche Diagnostics) and fresh Optimum medium (Invitrogen) was supplied 5 h after transfection. Apolipoproteins were added in the fresh medium at 10 μ g/ml. Conditioned medium was collected 24 h later. A β 40 or A β 42 was determined in triplicate using A β 40 or A β 42 ELISA Kit (Biosource International, Camarillo, CA). For studying the effect of PTB domain on A β production, N2a-APPsw cells were transfected with (0.4 μ g/per well for 24 well plate) PTB-containing vector before being subjected to the above procedure.

Internalization of cell surface proteins. N2a-APPsw cells were transiently transfected with ApoER2 and β -secretase or with ApoER2 and ADAM-10 using Lipofectamine 2000. Twenty-four hours after transfection, 10 μ g/ml ApoE or ApoE4 was added into medium and incubated for 2 h. Cells were then placed on ice, rinsed in cold PBS, and incubated in PBS containing 1.5 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL) for 20 min at 4°C. Cells were rinsed twice in PBS and lysed in 800 μ l of PBS containing 0.1% SDS, 1% NP40, and a complete protease inhibitor mixture. Total protein concentration was determined by immunoblotting from an aliquot of 50 μ l of cell lysate. Biotinylated proteins were recovered with NeutrAvidin agarose (50 μ l; Pierce), rinsed three times, eluted in 20 μ l of SDS sample buffer at boiling temperature, and used for Western blot. Quantity of bands was determined by optical scanning instruments specified above. For the determination of internalized proteins, cells were prepared as above, rinsed in PBS, and then incubated with cold 1.5 mg/ml cleavable biotin reagent in PBS (EZ-Link Sulfo-NHS-SS biotin; Pierce) for 20 min at 4°C. Cells were then rinsed quickly with medium at room temperature, incubated for 15 min at 37°C in control medium or in medium containing 10 μ g/ml ApoE or ApoE4, placed on ice, and rinsed in cold stripping buffer (50 mM glutathione, 75 mM NaCl, 75 mM NaOH, 10% FBS, pH 8.5–9.0). Cells were then lysed in 800 μ l of PBS containing 0.1% SDS, 1% NP40, and a complete protease inhibitor mixture. After centrifugation, biotinylated proteins were retrieved by incubation with NeutrAvidin agarose (50 μ l; Pierce). Isolated proteins were rinsed in buffer three times and boiled in 20 μ l of sample buffer for Western blots.

Immunofluorescence studies of cellular proteins. HeLa cells were seeded onto six-well plates with glass coverslips and expressed the ApoER2/X11 α , ApoER2/X11 β , or ApoER2/APP constructs for 36 h after transfection. Cells were gently fixed in 4% paraformaldehyde, in PBS, pH 7.4, at room temperature for 15 min. Coverslips were then washed twice for 10 min each in PBS and incubated for 1 h at room temperature with the indicated combinations of primary antibodies diluted in 0.1% BSA, 0.1% saponin, and 0.02% sodium azide in PBS (immunofluorescence buffer). At the end of this period, coverslips were washed twice with PBS followed by incubation for 30 min with the indicated combinations of secondary antibodies diluted in immunofluorescence buffer. Coverslips were again

washed twice with PBS and mounted on slides using Vectashield (Vector Laboratories, Burlingame, CA). APP was immunolabeled with Rabbit polyclonal antibody 5352 (Chemicon) and followed by cyanine 3 (Cy3)-conjugated sheep anti-rabbit secondary antibody (Sigma). X11 α and X11 β were blotted by monoclonal antibodies against them (BD Biosciences) and then recognized by Alexa 488-conjugated donkey anti-mouse secondary antibody (Invitrogen). ApoER2 was labeled either by rabbit polyclonal antibody against His-tag (colocalized with X11) or monoclonal antibody against V5 (colocalized with APP) and then was labeled by either Cy3 or Alexa 488-conjugated secondary antibodies. Images were obtained in an inverted confocal laser-scanning microscope (LSM 510; Zeiss, Oberkochen, Germany).

Small interfering RNA interference of ApoER2. Double-stranded small interfering RNA (siRNA) specific for the mouse ApoER2 (ON-TARGETplus SMARTpool, catalog number L-046407-00) was chemically synthesized by Dharmacon (Chicago, IL). N2a-APPsw cells grown in 24-well plates for 24 h were transfected with either ApoER2 siRNA or control siRNA (NO-TARGETplus siRNA; Dharmacon) with Oligofectamine (Invitrogen). The medium, Opti-MEM, was replaced 4 h later with ApoE or ApoE4 and cultured for 24 h. A β 40 in the medium samples were assayed as described above.

Studies on Reelin and Reelin effect. N2a-APPsw cells grown in a T25 flask were transfected with 5 μ g of expression vector pCrl containing the entire mouse Reelin gene using Lipofectamine 2000. Control cells were transfected with blank vector. Cells were conditioned in Opti-MEM for 36 h, and the medium was collected and concentrated. Western blot was performed on concentrated medium and cell lysate using anti-Reelin antibody MAB5364 (Chemicon). To determine the effect of Reelin antibody on A β production, N2a-APPsw cells were cultured for 24 h with 10 μ g/ml ApoE and either with or without antibody MAB5364. A β 40 in the medium was determined by ELISA.

Results

ApoE increases A β in N2a cells

We observed that A β production in Neuro-2a cells stably transfected with human APPswedish (N2a-APPsw) increased up to twofold in the presence of purified human VLDL ($p < 0.01$), whereas LDL and HDL had no effect (Fig. 1a). Because VLDL is the main lipoprotein fraction containing ApoE (Fig. 1a, inset), we tested the effect of each of the isomorphous ApoE forms and found that the addition of recombinant ApoE2, ApoE3, and ApoE4 to the cell culture at 10 μ g/ml increased A β production (Fig. 1b) with the highest response by ApoE4. Because these ApoE samples contained no lipids, the differential response may be influenced by their non-native physical properties. We therefore investigated whether ApoE4 from native human VLDL stimulates more A β production than either ApoE2 or ApoE3. VLDL was prepared from individual human plasma and the content of total ApoE and ApoE4 was determined. VLDL samples containing different levels of ApoE were chosen for mea-

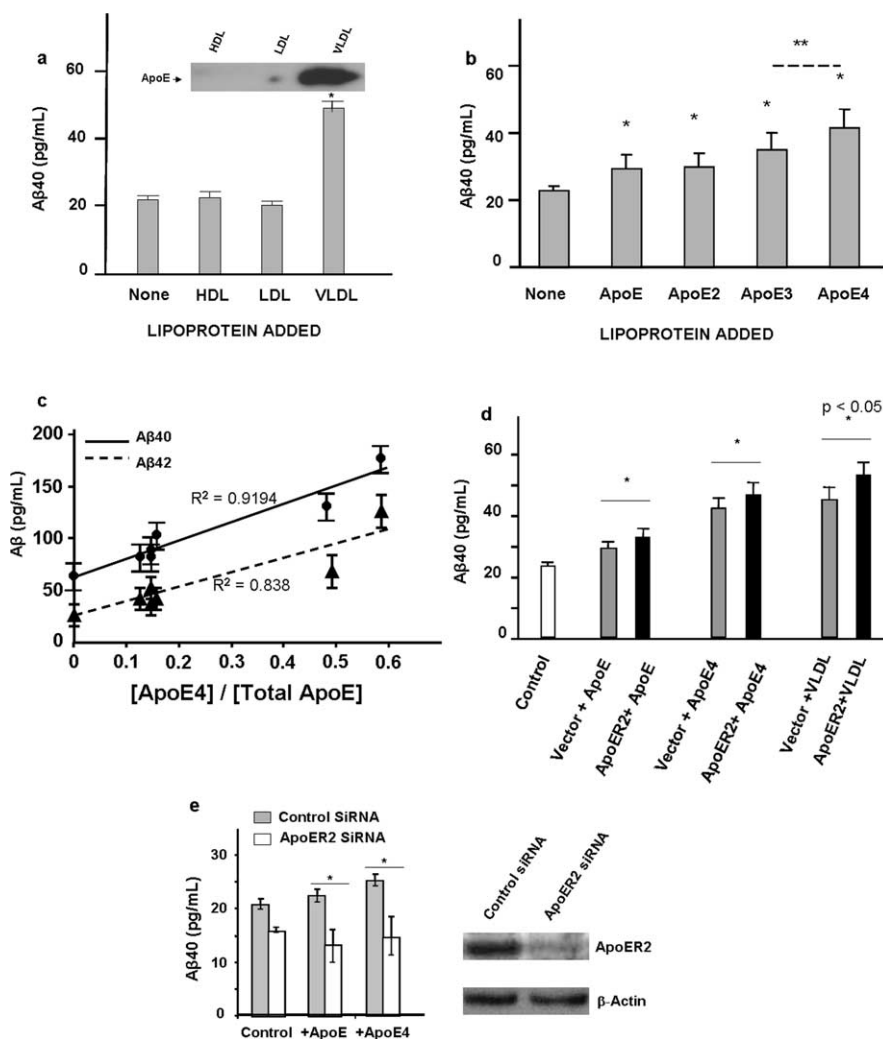


Figure 1. ApoE increases A β production in neuroblastoma N2a-APPsw cells. **a**, Human VLDL (15 μ g/ml) increased A β production in N2a-APPsw cells. HDL and LDL (same concentration) had no effect. A β in the medium was analyzed after 24 h of culture. Inset, Western blots of ApoE in three lipoprotein fractions. **b**, Purified human ApoE, recombinant ApoE2, ApoE3, and ApoE4 (each at 10 μ g/ml) increased A β production in N2a-APPsw cells. Experiment conditions are the same as in **a**. * $p < 0.05$; ** $p < 0.01$. **c**, Correlation of ApoE4 content of human VLDL with A β ₄₀ and A β ₄₂ production in N2a-APPsw cells. VLDL samples purified from individuals were analyzed for ApoE4 and total ApoE contents and measured with ELISA for their effect on A β ₄₀ and A β ₄₂ production in N2a-APPsw cells. **d**, Transfect of ApoER2 into N2a-APPsw cells increased ApoE or VLDL induced increase of A β production (black columns) over cells transfected with blank vectors (gray columns). The experimental conditions were the same as above. **e**, ApoER2 siRNA knockdown abolished ApoE- or ApoE4-induced increase of A β production (left). Western blots (right) show that the endogenous ApoER2 band was greatly reduced in ApoER2 siRNA knockdown, whereas the control β -actin band was not significantly changed.

suring the production of A β ₄₀ and A β ₄₂ in N2a-APPsw cells. We observed a linear correlation of ApoE4 content and the production of both A β species (Fig. 1c) with similar correlation coefficients. These results indicate that ApoE4 in native lipoprotein particles also stimulates higher A β production in N2a cells than those for ApoE2 and ApoE3.

ApoER2 mediates A β increase by ApoE

Because ApoER2 is a brain-specific receptor for ApoE, we asked whether the ApoE effect on A β is mediated by ApoER2. In the first set of experiments, the influence of ApoE and VLDL on A β production was compared for N2a-APPsw cells with or without transfection of ApoER2. Cells transfected with ApoER2 produced more A β in response to ApoE, recombinant ApoE4, or purified VLDL (Fig. 1d). These results suggest that ApoER2 mediated the increase of A β production in response to ApoE. ApoE also caused

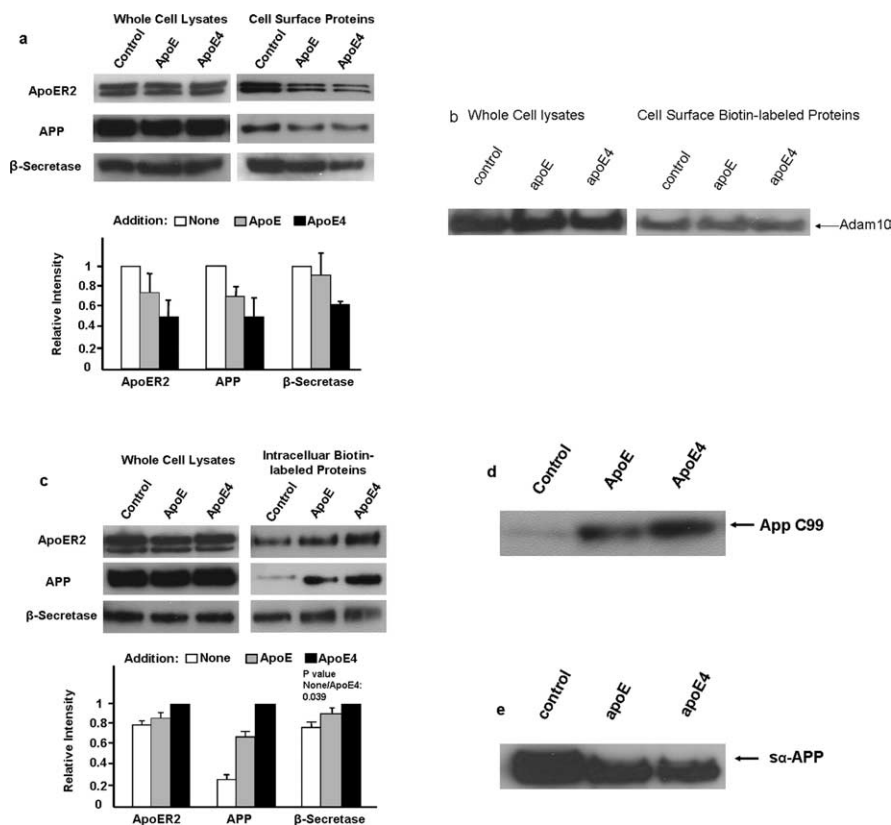


Figure 2. ApoE or ApoE4 induced the internalization of ApoER2, APP, and β -secretase from the cell surface. *a*, ApoE reduced cell surface ApoER2, APP, and β -secretase. N2a-APPsw cells transfected with ApoER2 and β -secretase were incubated with ApoE or ApoE4 for 2 h. Cell-surface proteins were biotinylated, retrieved with avidin–agarose gel, and subjected to Western blots for ApoER2, APP, and β -secretase (top right). Parallel experiments performed without biotinylation from total cell lysates were subjected to Western blots (top left). Quantitation of the bands was done by scanning densitometer and displayed here as relative intensity as the ratios of the surface protein to the total amount proteins (bottom). Controls (white columns) were set as 1.0 (average of 2 experiments). *b*, ApoE did not change cell-surface α -secretase (ADAM-10). N2a-APPsw cells transfected with bovine ADAM-10 and then cell-surface ADAM-10 was determined in the absence and presence of ApoE or ApoE4 using the same procedure and experimental conditions as described in *a*. An example of Western blots shows that neither ADAM-10 (HA-tag; right) nor the total cell lysates (left) was significantly changed in the presence of ApoE or ApoE4. *c*, ApoE increased intracellular ApoER2, APP, and β -secretase. Cells were prepared as in *a*, and then the cell-surface proteins were labeled with a cleavable biotinylation reagent at 4°C. The internalization was affected at 37°C for 15 min. After stripping the biotinyl group on cell surface, the cells were lysed, and the biotinylated proteins were retrieved for Western blots as above. The displayed panels are the same as in *a* except that at the bottom, the amounts of ApoE (black columns) were set as 1.0. *d*, Western blot of intracellular APP fragment C99 from N2a-APPsw cells in the absence and presence of ApoE. The samples were those described in *d*, blotted with antibody MAB1560. *e*, Western blot of soluble APP ectodomain generated by α -secretase (s-APP α) from N2a-APPsw cells in the absence and presence of ApoE. The condition medium from *a* was immunoprecipitated with MAB1560 and Western blotted with the same antibody.

A β to increase in cells transfected by blank vector (Fig. 1*d*), which provided an opportunity to study whether the increase was mediated by endogenous ApoER2. In the second series of experiments, we used siRNA to knock down ApoER2 and examined A β production in response to the various ApoE species. Figure 1*e* shows that only residual expression of endogenous ApoER2 remained in knockdown cells, which lost approximately one-fourth of their A β production and which did not increase after the addition of ApoE or ApoE4 to the media (Fig. 1*d*). These observations indicate that ApoER2 mediates ApoE-stimulated A β production in N2a-APPsw cells.

ApoE triggers the endocytosis of ApoER2, APP, and β -secretase

A possible explanation for ApoE stimulation of A β production is that ApoE triggers the internalization of not only ApoER2, but also APP and β -secretase from cell surface to endosomes. We

examined whether the addition of ApoE causes the reduction of these proteins from the cell surface. N2a-APPsw cells were treated with ApoE or ApoE4 for 2 h and then biotinylated to label the cell-surface proteins. Biotinylated ApoER2, APP, and β -secretase were recovered and revealed in Western blot. Compared with controls, the remaining cell-surface ApoER2, APP, and β -secretase decreased by ~25, 25, and 7%, respectively, when ApoE was added and 50, 50, and 35%, respectively, when ApoE4 was present (Fig. 2*a*). The total amounts of the three proteins in the cell lysates remained approximately the same. Under identical conditions, cell-surface ADAM-10 (known to be an α -secretase) was unchanged in the presence of either ApoE or ApoE4 (Fig. 2*b*), indicating the specificity of ApoE induced changes. We then determined whether ApoEs increased these three proteins inside the cells. Cell-surface proteins were pulse labeled by biotinylation and permitted to internalize for 15 min. Biotinyl groups remained at cell surface were stripped, and the biotinylated ApoER2, APP, and β -secretase inside the cells were retrieved and visualized in Western blot. We observed small increases of ApoER2 and β -secretase in response to ApoE and ApoE4 (Fig. 2*c*), whereas the increase of APP was much greater, ~2.5 and fourfold in response to ApoE and ApoE4, respectively (Fig. 2*c*). Together, the depletion of cell-surface ApoER2, APP, and β -secretase and the increase of the intracellular pool of these proteins in response to the addition of ApoE are consistent with the mechanism that the binding of ApoE to ApoER2 triggered the endocytosis of all three proteins. To further substantiate that the endocytosed APP was subjected to proteolysis by β -secretase, we also determined in the above experiments the APP C-terminal fragment resulting from

β -secretase cut (C99). We observed that C99 was significantly increased in the presence of ApoE and ApoE4 over the control (Fig. 2*d*). Again, ApoE4 generated more C99 than did ApoE. An increased endocytosis and decreased cell-surface APP stimulated by ApoE should be accompanied by a decrease of APP processing by α -secretase. Western blots of the APP ectodomain generated by α -secretase, s-APP α , confirmed a reduction of s-APP by ApoE and ApoE4 (Fig. 2*e*).

Role of X11 α/β in the ApoE and A β relationship

A hypothesis that may explain the ApoE triggered endocytosis of ApoER2 and APP is that their cytosolic domains may interact with a same adaptor protein that regulates cellular transport. The cytosolic domain of APP contains a YENPTY motif known to bind brain-specific adaptor proteins X11 α or X11 β (Fig. 3*a*). We seek to determine whether X11 α/β also interacts with ApoER2. When ApoER2 was immunoprecipitated from lysates of HEK293

cells transiently expressing ApoER2 and either X11 α or X11 β , the adaptor proteins were seen in the Western blots of the precipitate (Fig. 3*b*). Substitution of ApoER2 with LRP did not produce coimmunoprecipitation with X11 α or the ApoER2-binding PTB domain of X11 α (see below) (Fig. 3*c*), indicating that X11 binding to ApoER2 is specific rather than a general phenomenon with LDL receptor family. X11 α and X11 β gave essentially identical results. X11 γ was not tested because it is not expressed in the brain. In reverse pull-down, ApoER2 containing a His6 tag in the lysate collected with Ni-affinity gel also recovered X11 α/β (Fig. 3*d*). These results suggest that X11 α/β binds ApoER2 with high affinity. Because X11 α/β binds both ApoER2 and APP, we tested whether ApoER2 and APP form a complex in non-neuronal HEK293 cells devoid of these endogenous proteins of interest. When the lysates of cells expressing ApoER2, APP, and X11 α/β were immunoprecipitated with anti-APP antibody 5228, both APP and X11 were found in the Western blot of the precipitates (Fig. 3*e*). Again, both X11 α and X11 β supported the coprecipitation. Cells transfected with ApoER2 and APP but without X11 had virtually no coprecipitation of ApoER2 and APP (Fig. 3*e*). These results indicate that X11 α/β is required for the complex formation involving ApoER2, APP, and X11 α/β .

Domain interaction between ApoER2 and X11 α/β

PTB domain of X11 α but not the PDZ domain was shown to pull down ApoER2 from the cell lysate (Fig. 4*a*), indicating that it is responsible for ApoER2 binding. To identify the binding motif in ApoER2, various GST fusions from different regions of ApoER2 cytosolic domain were tested for X11 α/β pull down. Constructs containing the 59-residue insertion were able to bind X11 (Fig. 4*b*), indicating that this region contains an X11-binding motif. Various deletions or mutations were made for potential motif sites in the cytosolic domain of ApoER2. Deletions of a NPXY motif (Fig. 4*c*, mutants 1 and 2) or mutations of two PXXP motifs (Fig. 4*c*, mutants 3 and 4) did not affect the binding of X11–PTB domain to ApoER2. The deletion of residues YDRPLW (residues 899–904) within the 59-residue insertion abolished X11–PTB pull-down (Fig. 4*c*, mutant 5). This same deletion also abolished the pull-down of a separately expressed PTB domain of X11 α (Fig. 4*d*). These results indicate that the motif YDRPLW mediates the binding of ApoER2 to the PTB domain of X11 α/β .

X11 α/β involves in ApoE stimulated A β production

Because X11 α/β binds to both ApoER2 and APP and is required for the formation of a complex containing these three proteins

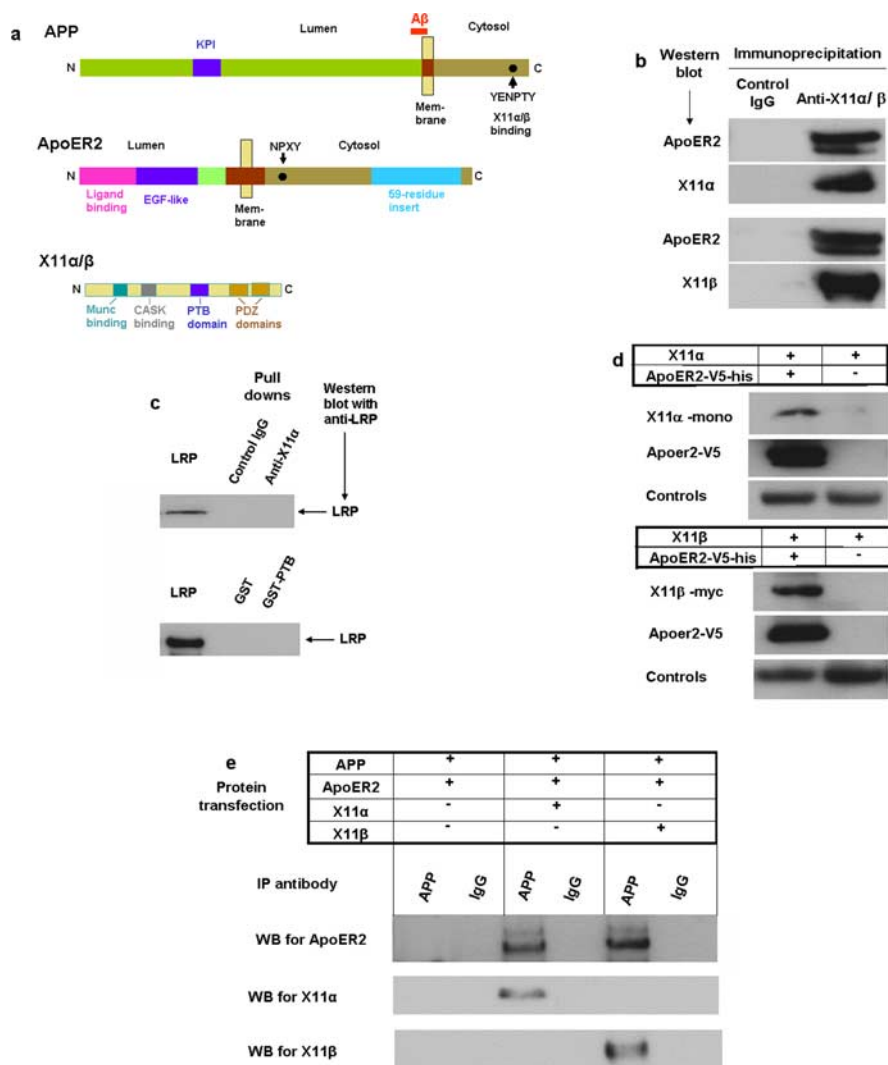


Figure 3. Binding of X11 α/β with ApoER2. **a**, Diagrams of some functional domains of APP, ApoER2, and X11. In APP, a dot denotes the location of the YENPTY motif, which involves in binding of several adaptor proteins including X11 α/β . KPI is a Kunitz protease inhibitor domain. In ApoER2, the alternative spliced 59-residue insertion is shown in blue. N, N terminus; C, C terminus. **b**, ApoER2 is recovered in immunoprecipitation of X11 α/β . Lysates of HEK293 cells transiently expressing ApoER2 (with V5 tag) and X11 α or X11 β (with myc tag) were immunoprecipitated separately with anti-X11 α , anti-myc (for X11 β), or control IgG and visualized in Western blots with anti-V5, anti-X11 α , or anti-myc. ApoER2 band was present in both immunoprecipitation for X11 α and X11 β . **c**, LRP did not coimmunoprecipitate with X11 α . Experimental conditions are the same as in **a**, **d**. Presence of X11 α/β in ApoER2 pull-down experiments. Lysates of cells expressing ApoER2 (with both V5 and hexahistidine tags) and X11 α/β were subjected to binding by Ni-affinity gel to retrieve ApoER2. Western blots showed that both X11 α (top) and X11 β (bottom) were recovered. **e**, ApoER2 and X11 α/β are immunoprecipitated (IP) with APP. Lysates of HEK293 cells transiently expressing APP, ApoER2, and X11 α or X11 β were immunoprecipitated with anti-APP antibody 5228 and visualized by Western blots (WB). ApoER2 was present when X11 α/β was expressed (lanes 3 and 5) but was absent when X11 was not expressed (lane 1).

(Figs. 3, 4), we hypothesize that it is involved in facilitating endocytosis of these proteins leading to ApoE-induced A β production. This hypothesis is supported by the intracellular colocalization of APP with ApoER2 (Fig. 5*A*, *a–c*) and ApoER2 with X11 α/β (Fig. 5*A*, *d–i*), especially in subcellular compartments consistent with endosomes (Fig. 5*A*, arrows). However, it is also known (Borg et al., 1998; Sastre et al., 1998) and we have confirmed (results not shown) that the expression of X11 α/β inhibits A β production. Such a response seems to contradict with the hypothesis. Therefore, we seek evidence on the involvement of endogenous X11 α/β in the ApoE-induced A β increase from N2a cells. First, transfected PTB domain from X11 α was used as dominant-negative inhibitor for the function of endogenous

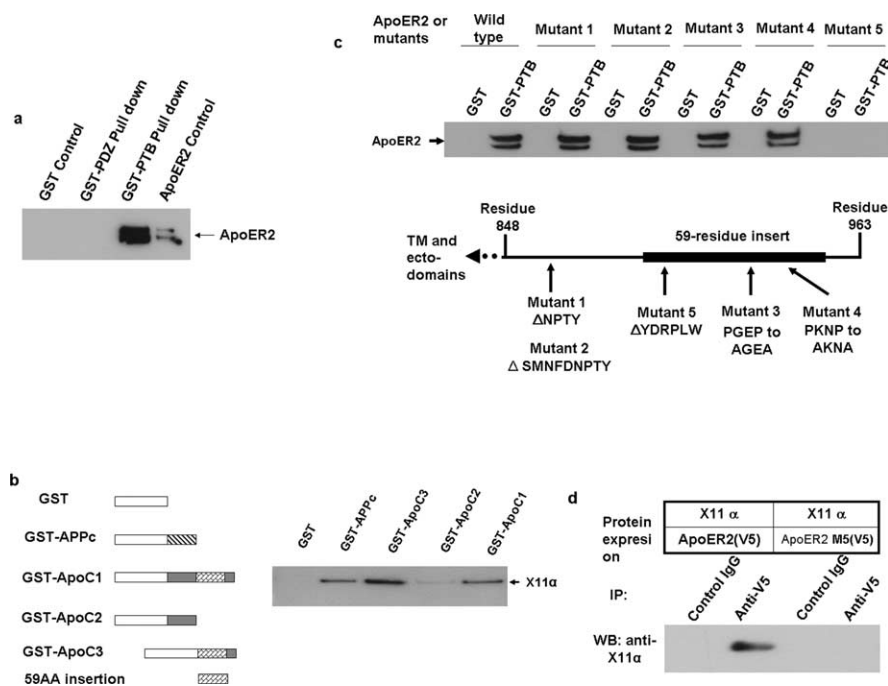


Figure 4. Domains and motif involved in ApoER2/X11 α / β interaction. **a**, PTB domain of X11 α binds ApoER2. Lysates from cells expressing ApoER2 and GST (glutathione-S-transferase) fusions of PTB or PDZ domains of X11 α were subjected to GST pull down using a glutathione affinity column. Western blots showed that ApoER2 was associated with PTB domain (third lane) but not PDZ domains (second lane). **b**, X11 α pull-down by GST (white bar) fusion constructs containing different regions of ApoER2 intracellular regions (gray bar). GST fusion to APP cytosolic domain (slash bar) was the positive control. Constructs containing the 59-residue insertion (check bar) in GST-ApoC1 and GST-ApoC3 were able to pull down X11 α from cell lysate, as shown in the Western blot. **c**, GST-PTB (X11 α) pull-down of ApoER2 mutants as shown by Western blot (top). The bottom shows a diagram of mutation positions. Deletion of the NPTY motif (mutants 1 and 2) or mutation of a PXXP motif (mutants 3 and 4) did not affect the pull-down. Only the deletion of the YDRPLW motif abolished the pull-down. GST was the negative control in pull-down and Western blot. **d**, Reversed pull-down from **d**, in which wild-type ApoER2 pulled down X11 α (lane 2), but mutant 5 (ApoER2 M5) did not (lane 4). IP, Immunoprecipitation; WB, Western blot.

X11 α / β . We observed that ApoE4-induced A β increase (Fig. 5B, compare columns 1 and 3) was largely abolished by PTB expression (Fig. 5B, column 4). Second, the expression of mutant ApoER2 with a deletion of the X11 α / β -binding motif (Fig. 4c, mutant 5) also greatly reduced A β increase induced by ApoE4 (Fig. 5C). These observations suggest that X11 α / β binding at least to ApoER2 plays a role in ApoE-induced endocytosis leading to the production of A β .

ApoER2-mediated A β increase by ApoE is independent of Reelin

ApoER2 is also a receptor for the neuronal signaling protein Reelin (D'Arcangelo et al., 1999; Hiesberger et al., 1999), which is known to reduce cellular A β production (Hoe et al., 2006). It is of interest then to determine whether ApoER2-mediated A β increase by ApoE is related to Reelin–ApoER2 interaction. Western blots of N2a-APPsw culture medium or cell lysate concentrated from cell culture in T25 flasks did not detect the presence of Reelin (Fig. 6a, two left lanes), whereas the Reelin bands were clearly visible in cells transfected with a Reelin expression vector (Fig. 6a, two right lanes). Moreover, the addition of anti-Reelin antibody to the culture medium did not significantly alter the ApoE-stimulated A β increase in N2a-APPsw cells (Fig. 6b). These observations indicate that ApoER2-mediated A β increase by ApoE is independent of Reelin–ApoER2 interaction.

Discussion

Data presented above suggest that ApoE induces the production of A β through a mechanism as follows (Fig. 7). At cell surface,

ApoER2 and APP are part of a complex mediated by X11 α / β . After the binding of ApoE to ApoER2 in the complex, the entire complex, along with β -secretase, is endocytosed. The hydrolysis of APP by β -secretase and the subsequent generation of A β by this process take place in acidic intracellular compartments, such as endocytic vesicles and endosomes, as is consistent with the observation of ApoE-induced increase of intracellular C99 (Fig. 2d).

The observation that ApoE4 generates more A β than does ApoE2 or ApoE3 in an ApoER2-mediated system (Fig. 1) suggests that the ApoE4 may bind ApoER2 more intensely than ApoE2 or ApoE3. Three isomorphous forms of ApoEs differ not only in two residues but also in conformations (for review, see Y. Huang et al., 2004); thus, their binding to receptors could be significantly different. Although we used plasma VLDL and purified ApoE to reach the above conclusions, these observations are likely applicable to brain lipoproteins despite the difference of its density from that of plasma VLDL. Although the density is determined by the lipid content in the interior of the lipoprotein particles, ApoE at the surface of the particles should interact in the same manner to ApoER2, thus triggering the same downstream events as shown in Figure 7. This is supported by the fact that we observed the same responses with isomorphous ApoEs in VLDL and in lipid-free form. Therefore, the current observations suggest that the ApoER2-mediated A β production may play a role in linking ApoE polymorphism with the risk of the sporadic form of AD. Other mechanisms that link ApoE4 to AD risk have been reported. For example, the association of isomorphous forms of ApoE with LRP, another member of the LDL receptor family, has been shown to affect the efflux of A β from the brain (Tanzi et al., 2004). ApoE-stimulated increase of A β mediated by LPR has also been reported recently (Ye et al., 2005). Although the underlying mechanism of this association is unknown, it seems possible that a mechanism similar to the one discussed here is also operative for LPR. However, ApoER2 knockdown completely abolished A β response to ApoE4, suggesting the absence of a parallel system in N2a cells. The fact that A β ₄₀ and A β ₄₂ respond to ApoE in a similar manner (Fig. 1c) suggests that the effect of an ApoE-triggered mechanism does not significantly influence the hydrolysis of C99 by γ -secretase. The proposed mechanism is also indirectly supported by the reduction of α -APP by ApoE species (Fig. 2e) because the increased APP endocytosis leads to a reduction of APP available at the cell surface for the activity of α -secretase. We observed that ApoE4 was more effective than ApoE in the increase of APP endocytosis and decrease of α -APP. A previous report described ApoE3-increased and ApoE4-decreased α -APP in PC12 cells (Wolozin et al., 1996). Although PC12 is a neuronal cell line, the different ApoE4 effects could be derived from a difference in ApoER2 or X11 α / β level from that in N2a-APP cells. Other possibilities include the presence of other ApoE-driven mechanisms in PC12.

How X11 α/β bridges ApoER2 to APP is unclear. X11 α/β has only one PTB domain, which binds ApoER2 or APP. Although an X11 homolog in *Caenorhabditis elegans* (lin-10) has been shown to form a dimer (Walhout et al., 2000), it is uncertain that this can be extended to X11 α/β . It seems plausible that the PTB domain of different X11 α/β molecules binds to APP and ApoER2 separately (Fig. 7). The complex is likely formed in the lipid raft at cell surface as a prelude to endocytosis. Considering the complexity of the endocytic assembly, it seems probable that other cytosolic proteins are involved in the formation of this complex. As an adaptor protein with multiple binding domains, it is reasonable to assume that the bound X11 α/β may interact with other partners (Fig. 7, question mark) to facilitate the recruitment and packaging of the endocytic vesicles. The clarification of the detailed process will require additional studies. The binding of ApoE-containing lipoprotein particles to ApoER2 triggers endocytosis of not only ApoER2 and APP but also β -secretase (Fig. 2). The coordinated internalization of APP can be explained by the X11 α/β -mediated complex formation. The coordinated endocytosis of β -secretase is particularly interesting, because it did not immunoprecipitate with the ApoER2 complex (results not shown). We previously reported that APP mediates the endocytosis of ectodomain of β -secretase (X. P. Huang et al., 2004), suggesting that β -secretase is recognized by the network of the APP-containing endocytic assembly at cell surface. This association does not involve the catalytic site of the protease (X. P. Huang et al., 2004) and at the pH of the lumen, the protease has little activity, thus the complex should be stable before reaching the acid intracellular compartments. Such association is consistent with the current observations and could play a role in the coordinated internalization, although we have not provided evidence of its specific involvement. The results in Figure 2 may be interpreted as the amount of internalization is in the order of APP, ApoER2, and β -secretase. However, these data were collected at a single time point, and a firm conclusion on relative internalization rates would require more detailed kinetic studies. The scheme in Figure 7 can also explain the inhibition of A β production by the expression of X11 α/β (Borg et al., 1998; Sastre et al., 1998). In the absence of ApoE, extra amounts of X11 α/β would keep a larger fraction of cell-surface APP in a complex involving ApoER2; thus, less APP is available for internalization and A β generation by alternative mechanisms.

The scheme in Figure 7 can be thought of as a triggering mechanism for the production of A β . In the absence of the triggering of ApoE, X11 α/β actually inhibits A β production. In the presence of ApoE, ApoER2, and X11 α/β facilitate A β production. This

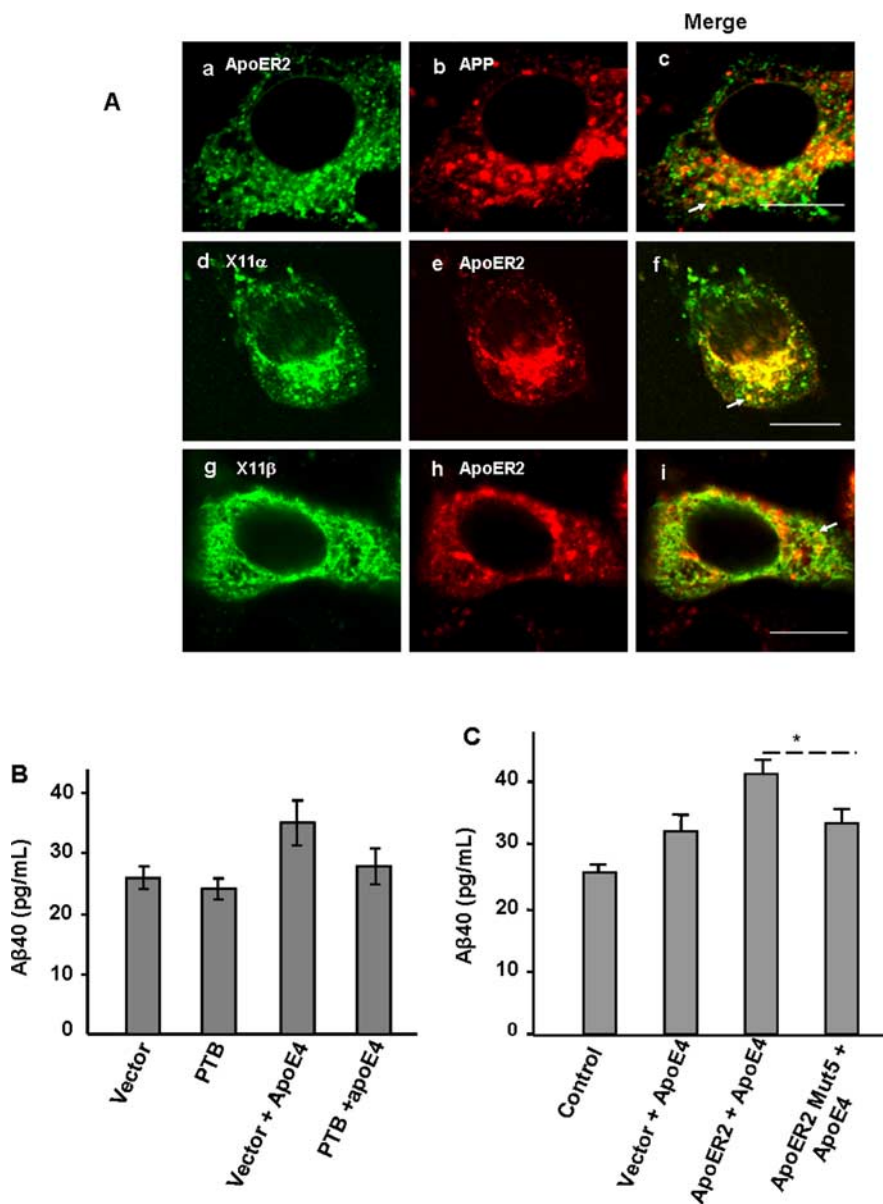


Figure 5. Involvement of X11 α/β in APP and ApoER2 internalization and ApoE-induced A β production. *A*, Intercellular localization of APP with ApoER2 (*a–c*) and ApoER2 with X11 α/β (*d–f*). Arrows in the merged images point to subcellular compartments consistent with endosomes. Scale bar, 10 μ m. *B*, Expression of PTB domain of X11 α/β abolished ApoE-induced A β production. *C*, Deletion of X11 α/β -binding motif YDRPLW in ApoER2 (Mut5; mutant 5 in Fig. 4c) abolished ApoE-induced A β production (* $p < 0.05$).

mechanism provides a link between the neuronal uptake of lipid particles and A β production. The relationship of lipid, especially cholesterol, level to A β production and AD has been well documented in cellular and animal models and in clinical trials (for review, see Puglielli et al., 2003; Wolozin, 2004). The mechanism described here may conceivably play a role in this pathogenic relationship. The physiological function of such a link may also be important. ApoE is the major lipoprotein carrier in the brain for the delivery of lipids, predominantly cholesterol, to neurons (Huang, 2006). Such a process is initiated on cell surface by the binding of ApoE to LDL receptor family, including ApoER2, followed by endocytosis. Cholesterol and lipids supplied in this process are essential in remodeling neuronal membranes and developing new growth terminals. These processes are important to neuronal activities and synaptic plasticity. Thus, it is logical that

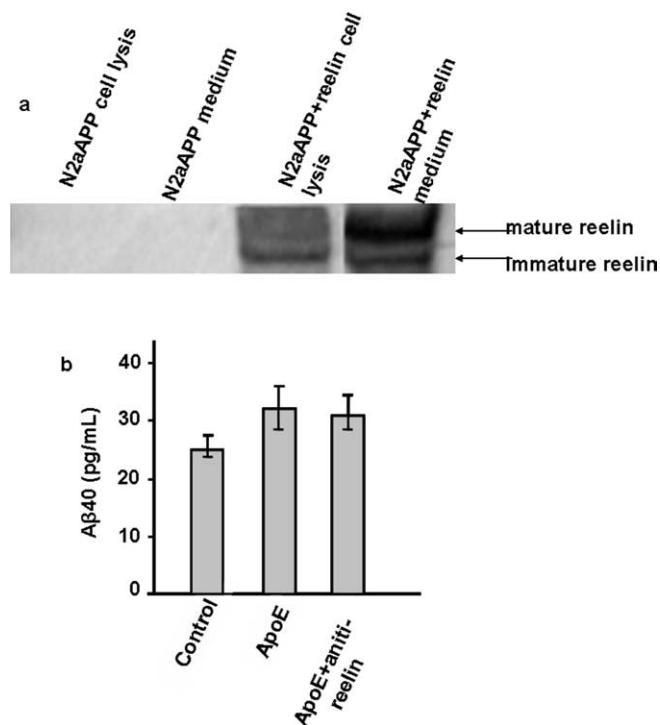


Figure 6. Absence of Reelin and a Reelin effect in ApoE triggered A β increase in N2a-APPsw cells. *a*, Western blots for Reelin in cell lysate and culture medium of N2a-APPsw cells (left two lanes) and N2a-APPsw cells transfected with a Reelin expression vector. *b*, A β 40 production in N2a-APPsw cells in the presence (right column) and absence of anti-Reelin antibody (left and center columns). Values were averaged from three determinations.

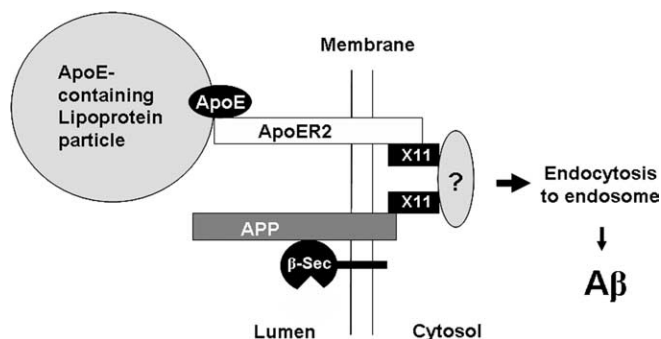


Figure 7. A schematic presentation of ApoE-triggered A β production. At the cell surface, ApoER2 (white) and APP (gray) each bind a molecule of X11 α / β (green) to form a complex with the possible involvement of other cytosolic proteins (black and ?) during the recruitment and packaging of endocytic vesicles. β -Secretase (β -Sec) is associated with this complex by virtue of its recognition of APP, in which the protease is inactive at pH 7. In the presence of ApoE-containing lipid particles, ApoE binds ApoER2 to trigger endocytosis of the complex with β -secretase to intracellular compartments in which, at pH 4.5, APP is cleaved by β -secretase and γ -secretase to generate A β . In this mechanism, ApoE triggers the production of A β , in which ApoE4 generates more A β than do ApoE2 and ApoE3, possibly as a result of a stronger association with ApoER2. This mechanism also links A β production to lipid uptake, which may be associated with neuronal activities.

expect that high neuronal activity is associated with the uptake of ApoE-coated lipid particles, the predominant supplier of lipids in the brain. Kamenetz et al. described a mechanism for the physiological function of A β in which high neuronal activity leads to an enhanced β -secretase activity and A β production. A β then feedback inhibits synaptic activity as a downregulation mechanism of neuronal functions (Kamenetz et al., 2003). The scheme in Figure 7 is consistent with the mechanism proposed by Kame-

netz et al. In linking these two mechanisms, high neuronal activity leads to ApoE-triggered endocytosis of APP and β -secretase; then, the increased A β production affects the feedback inhibition. The possible connection of these two mechanisms warrants additional investigation.

Our data from N2a cells show that the mechanism in Figure 7 is independent of Reelin. The increase of A β production by ApoE can be accounted for from the increased endocytosis shown in Figure 2 and does not involve a mechanism of reversing A β inhibition of Reelin by ApoE. The absence of Reelin in N2a cells (Fig. 6*a*) is not surprising because Reelin is produced specifically by Cajal-Retzius neurons. As discussed above, the current mechanism in Figure 7 is likely to be a general one that links neuronal functions to A β production, whereas Reelin signaling appears to have more regulatory functions. Our results do not rule out, however, that Reelin and ApoE do compete for ApoER2 under Reelin signaling conditions as suggested by previous studies (D'Arcangelo et al., 1999; Hiesberger et al., 1999).

ApoER2 motif (mouse, YDRPLW; human, FDRPLW) recognized for X11 α / β binding is only moderately related to that in APP (YENPTY). Another similar motif (FDNPVY) in the cytosolic domain of ApoER2 is not recognized by X11 α / β , perhaps because of the presence of a valine. These observations suggest a consensus X11 α / β -binding sequence with F/Y at residue 1, E/D at residue 2, P at residue 4, and a hydrophobic residue at residue 6 of the motif. This motif is located in the 59-residue insertion (Fig. 3*a*) and resulted from a brain-specific alternative splicing of exon 19, a region known to modulate synaptic plasticity and learning (Beffert et al., 2005). Our observation that the motif in this region binds X11 α / β , leading to the production of A β , which in turn regulates the synaptic activities, is consistent with such a function.

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