Apolipoprotein E4 Domain Interaction Occurs in Living Neuronal Cells As Determined by Fluorescence Resonance Energy Transfer*

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1The abbreviations used are: AD, Alzheimer’s disease; apo, apolipoprotein; CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; YFP, yellow fluorescent protein.
Apolipoprotein (apo) E4 is a major risk factor for Alzheimer’s disease (AD). Although the mechanisms remain to be determined, the detrimental effects of apoE4 in neurobiology must be based on its unique structural and biophysical properties. One such property is domain interaction, mediated by a salt bridge between Arg-61 in the N-terminal domain and Glu-255 in the C-terminal domain of apoE4. This interaction, which does not occur in apoE3 or apoE2, causes apoE4 to bind preferentially to certain lipoprotein particles \emph{in vitro} and \emph{in vivo}. Here we used fluorescence resonance energy transfer (FRET) to determine if apoE4 domain interaction occurs in living neuronal cells. Neuro-2a cells were transfected with constructs encoding apoE3 or apoE4 in which yellow fluorescent protein (YFP) was fused to the N-terminus and cyan fluorescent protein (CFP) to the C-terminus. To generate a FRET signal that can be detected by spectrum confocal microscopy, the labeled N- and C- termini must be in close proximity (<100 Å). FRET occurred in cells transfected with YFP-apoE4-CFP, but not in those transfected with YFP-apoE3-CFP, suggesting that the N- and C-termini of apoE4 are in close proximity in living cells, and those of apoE3 are not. FRET did not occur in cells cotransfected with YFP-apoE4 and apoE4-CFP, suggesting that the FRET in YFP-apoE4-CFP-transfected cells was intramolecular. Mutation of Arg-61 to Thr or Glu-255 to Ala in apoE4, which disrupts domain interaction, abolished FRET in Neuro-2a cells, strongly suggesting that the FRET in YFP-apoE4-CFP cells was caused by domain interaction. ApoE4-producing cells secreted less phospholipid than apoE3-producing cells, but after disruption of domain interaction in apoE4, phospholipid secretion increased to the levels seen with apoE3, suggesting that domain interaction decreases the phospholipid-binding capacity of apoE4. Thus, apoE4 domain interaction occurs in living neuronal cells and might be a molecular basis for apoE4-related neurodegeneration.
Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by progressive dementia (1, 2). One isoform of human apolipoprotein (apo) E, apoE4, is a major risk factor for AD (3-8). ApoE4 is found in neurofibrillary tangles and amyloid plaques—two neuropathological hallmarks of AD (2, 3, 9-13). Although, its pathogenic role in these lesions is still poorly understood, apoE4 has several adverse effects that might explain its association with AD. It modulates the deposition and clearance of amyloid-β peptides and plaque formation (14-20), impairs the antioxidative defense system (21), dysregulates neuronal signaling pathways (22), disrupts cytoskeletal structure and function (23, 24), alters the phosphorylation of tau and the formation of neurofibrillary tangles (25-28), and potentiates lysosomal leakage and apoptosis induced by amyloid-β peptides in neuronal cells (29). The mechanisms of these effects are still largely unknown, and it is not known whether they result directly or indirectly from apoE activities or to what extent they contribute to the pathogenesis of AD-related dementia.

Since the structural and biophysical properties of a protein determine its function, the detrimental effects of apoE4 in neurobiology must reflect properties unique to apoE4. ApoE3 and apoE4 have identical amino acid sequences except at position 112, where apoE4 has arginine and apoE3 has cysteine (30-33). This difference results in fundamental structural and biophysical differences (30-34). For example, apoE4 displays domain interaction, mediated by a salt bridge between Arg-61 in the N-terminus and Glu-255 in the C-terminus, leading to a compact structure (35, 36). As a result, apoE4 binds preferentially to triglyceride-rich very low density lipoproteins (35, 36). ApoE3, which lacks the domain interaction, is predicted to have a more open structure and binds preferentially to phospholipid-rich high density lipoproteins (35, 36). ApoE4 domain interaction occurs on lipoprotein particles in vitro in human plasma (35, 36) and in vivo in Arg-61 knock-in mice (37), in which domain interaction was introduced into mouse apoE by mutating Thr-61 to Arg.
Although it was initially thought that apoE was primarily synthesized by astrocytes in the brain, but not by neurons (38), numerous subsequent studies have demonstrated that central nervous system neurons also express apoE, albeit at lower levels than astrocytes, under diverse physiological and pathological conditions (39-54). Notably, transgenic mice expressing apoE4 specifically in central nervous system neurons are more susceptible to age- and excitotoxin-induced neurodegeneration (55-57) and behavioral deficits (55, 58) than transgenic mice with similar expression of apoE3. Domain interaction may contribute to the neurodegenerative effects of apoE4 in those transgenic mice (31, 34, 59, 60). However, domain interaction has not been demonstrated in living neuronal cells.

To explore this fundamental question, we used fluorescence resonance energy transfer (FRET) to analyze living neuronal cells expressing apoE3 or apoE4. FRET—the nonradiative transfer of photon energy from an excited fluorophore (donor) to another fluorophore (acceptor)—occurs only when the donor and acceptor are in close proximity (<100 Å). Thus, this approach can be used to measure nanometer scale distances. It has been used to study domain reorganization induced by lipid association in human apoE3 (61, 62). Recent advances, such as new fluorescent probes and new imaging methods, have made it possible to use this technique to detect protein–protein interactions and changes in protein conformation in living cells (63-69). For example, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) can be used to label two proteins to detect intermolecular interactions or to label two termini of a protein to detect intramolecular domain interaction in living cells by measuring FRET (63-74).

In the current study, we used FRET to examine apoE4 domain interaction in living neuronal cells expressing apoE3 or apoE4 fused to fluorescent proteins (YFP-apoE3-CFP and YFP-apoE4-CFP). Our results demonstrate that domain interaction of apoE4 occurs in living neuronal cells, which might be a molecular basis for apoE4-related neurodegeneration.
EXPERIMENTAL PROCEDURES

Materials—Minimum essential medium, N2-medium supplements, and fetal bovine serum were purchased from Life Technologies (Rockville, MD). The ECL chemiluminescence detection kit for Western blots was from Amersham Life Science (Piscataway, NJ). Monoclonal anti-FLAG (M2) was from Sigma. Horseradish peroxidase–coupled anti-goat IgG was from Dako (Carpinteria, CA).

Preparation of cDNA Constructs Encoding ApoE3 or ApoE4 Fused with YFP and CFP—PCR products encoding wildtype or mutated (Arg-61 to Thr or Glu-255 to Ala) human apoE3 or apoE4 without a stop codon were subcloned into a pFLAG-CMV3 vector (Sigma) that contains an amino-terminal FLAG tag and a signal peptide sequence. A PCR product encoding YFP without a stop codon was amplified from the pEYFP-N1 vector (Clontech, Palo Alto, CA) and subcloned into the pFLAG-CMV3-apoE3, pFLAG-CMV3-apoE4, pFLAG-CMV3-apoE4-Thr-61, or pFLAG-CMV3-apoE4-Ala-255 vector at the N-terminus of apoE. Finally, a PCR product encoding CFP with a stop codon was amplified from the pECFP-C1 vector (Clontech) and subcloned into the pFLAG-CMV3-YFP-apoE3, pFLAG-CMV3-YFP-apoE4, pFLAG-CMV3-YFP-apoE4-Thr-61, or pFLAG-CMV3-YFP-apoE4-Ala-255 vector at the C-terminus of apoE. cDNA constructs encoding YFP-apoE3-CFP, YFP-apoE4-CFP, YFP-apoE4-Thr-61-CFP, or YFP-apoE4-Ala-255-CFP were generated. All DNA constructs were confirmed by sequence analysis.

Cell Cultures and Transfection—Mouse neuroblastoma Neuro-2a cells (American Type Culture Collection) were maintained at 37 °C in minimum essential medium containing 10% fetal bovine serum. Neuro-2a cells were transiently transfected with the apoE cDNA constructs described above using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) (27).

Western Blotting—Neuro-2a cells were harvested 24 h after transfection with various apoE cDNA constructs and lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl,
0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and Roche complete protease inhibitors) for 30 min. After centrifugation at 13,000 rpm for 15 min in an Eppendorf centrifuge (Westbury, NY), apoE labeled with both YFP and CFP was detected in the supernatant by Western blotting with M2 monoclonal antibodies against the FLAG tag (Sigma) (75).

**Imaging FRET by Multiphoton Confocal Microscopy**—Neuro-2a cells transiently transfected with the YFP-apoE3-CFP or YFP-apoE4-CFP cDNA construct were grown in serum-free minimum essential medium for 18–20 h, illuminated with a Zeiss two-photon laser, and examined with a Zeiss confocal microscope. To visualize CFP fluorescence, transfected cells were excited at 458 nm, and images were acquired at 465–512 nm. FRET images were acquired at the emission wavelength of YFP (543–618 nm) when CFP was excited (66, 67, 76, 77). Although YFP and CFP have broad absorption and emission peaks that partially overlap, the Meta Detector of the Zeiss multiphoton confocal system can deconvolve the contribution of each fluorochrome to each pixel by spectral analysis using information obtained from the reference spectra (YFP or CFP alone). Thus, we could image YFP and CFP fluorescence simultaneously in neurons expressing YFP-apoE3-CFP or YFP-apoE4-CFP at similar levels for FRET analysis.

**Quantifying FRET in Transfected Neuro-2a Cells**—Transiently transfected Neuro-2a cells expressing YFP-apoE3-CFP or YFP-apoE4-CFP at similar levels were selected by fluorescence microscopy. YFP and CFP images of transfected cells were acquired with the Meta Detector, and their fluorescence intensities were analyzed with the mounted computer. The FRET signal was calculated as the ratio of YFP to CFP fluorescence intensity under CFP excitation (65-67, 78, 79). For each YFP-apoE-CFP construct, the FRET signal was measured in at least 12 cells from different fields.

**Detecting FRET by Photobleaching YFP Fluorescence in Transfected Cells**—After the CFP images were acquired and their fluorescence intensities were calculated, a strong laser (514 nm, 100% power, eight 10-sec cycles) was used to photobleach YFP fluorescence in cells expressing
YFP-apoE3-CFP, YFP-apoE4-CFP, or both YFP-apoE4 and apoE4-CFP. CFP images were then acquired, and their fluorescence intensities were recalculated. An increase in CFP fluorescence intensity after photobleaching of YFP fluorescence indicates FRET (66, 73, 80). For each experiment, more than six fields of the slides were photobleached.

Quantifying FRET in the Culture Medium—Neuro-2a cells stably expressing YFP-apoE3-CFP or YFP-apoE4-CFP and wildtype cells were grown in T175 flasks to 90% confluence and incubated with serum-free minimum essential medium containing N2 supplement for 24 h. The conditioned medium (20 ml/flask) was concentrated about 20-fold with Centriplus-YM-10 concentrators (Amicon, Bedford, MA), dialyzed against PBS, illuminated at the CFP excitation wavelength (430 nm), and scanned for emission spectrum. The FRET signal was calculated as the ratio of emission at 525 nm (YFP) to that at 475 nm (CFP). Conditioned medium from cells expressing apoE3-CFP or apoE4-CFP was used to determine baseline fluorescence in the absence of FRET.

Determining the Lipid Content of ApoE-containing Lipoproteins in Culture Medium—Cells transiently or stably expressing apoE3, apoE4, YFP-apoE3-CFP, YFP-apoE4-CFP, or YFP-apoE4-Thr-61-CFP were incubated in T175 flasks containing serum-free minimum essential medium with N2 supplement (20 ml/flask) for 24 h. After concentration as described above, 100 µl of conditioned medium was incubated with 3 µl of anti-apoE IgG (Calbiochem, San Diego, CA) at 4 °C overnight and then with 60 µl of protein-A agarose beads (Bio-Rad, Hercules, CA) at 4 °C for 4 h and centrifuged at 6000 x g for 5 min to precipitate apoE-containing lipoprotein particles. To confirm complete precipitation of apoE, the supernatant was subjected to anti-apoE Western blotting. The concentrations of phospholipid, cholesterol, and triglyceride in the medium before and after immunoprecipitation were determined as described (81). The amount of lipids bound with various forms of apoE was calculated by subtracting the value obtained after anti-apoE immunoprecipitation from that obtained before immunoprecipitation. The levels of
apoE3, apoE4, YFP-apoE3-CFP, YFP-apoE4-CFP, or YFP-apoE4-Thr-61-CFP in the conditioned medium were measured semiquantitatively by anti-apoE Western blotting (81) and used to normalize the lipids bound with various forms of apoE.

Statistical Analysis—Results are reported as mean ± SD. Differences were evaluated by t test or analysis of variance.
RESULTS AND DISCUSSION

To determine if apoE4 domain interaction occurs in living neuronal cells, we prepared cDNA constructs encoding YFP-apoE3-CFP or YFP-apoE4-CFP (Fig. 1A). Control cDNA constructs encoding YFP-apoE3, apoE3-CFP, YFP-apoE4, or apoE4-CFP were also prepared. All of these fusion proteins have a FLAG tag at the N-terminus. After transient transfection of mouse neuroblastoma Neuro-2a cells with these constructs, all of the singly and doubly labeled forms of apoE were detected at similar levels in cell lysates (Fig. 1A) and medium (data not shown) by Western blotting with monoclonal anti-FLAG.

Since the emission spectrum of CFP (460–520 nm) overlaps with the excitation spectrum of YFP (480–520 nm), spectrum confocal microscopy can be used to measure FRET in living cells (70, 71). If CFP and YFP are in close proximity (<100 Å), part of the emission energy under CFP excitation is transferred from CFP to YFP, thereby increasing YFP emission and decreasing CFP emission. Thus, the occurrence of FRET in transfected Neuro-2a cells expressing double-labeled apoE indicates close proximity of the N- and C-termini, consistent with domain interaction in apoE4; the absence of FRET or a very weak FRET signal suggests that the two termini are farther apart, consistent with the lack of domain interaction in apoE3 (Fig. 1B).

FRET Occurs Only in Neuro-2a Cells Expressing YFP-apoE4-CFP—We used three approaches to detect FRET in transfected Neuro-2a cells. First, we imaged YFP fluorescence under CFP excitation (66, 67, 76, 77). A strong FRET signal was detected in Neuro-2a cells expressing YFP-apoE4-CFP (Fig. 2), suggesting that the N- and the C-termini of apoE4 are <100 Å apart, presumably due to domain interaction. However, very weak (or background) FRET (YFP) fluorescence was detected in cells expressing YFP-apoE3-CFP, suggesting that the two termini of apoE3 are separated by >100 Å, indicating a lack of domain interaction.

Second, we determined the ratio of FRET (YFP) to CFP fluorescence as a measurement of the FRET intensity in Neuro-2a cells expressing YFP-apoE3-CFP or YFP-apoE4-CFP (65-67,
When FRET occurs under excitation of CFP, YFP emission increases and CFP emission decreases, reflecting energy transfer from CFP to YFP (Fig. 3A). Therefore, the ratio of FRET (YFP) to CFP fluorescence can be used as a measure of FRET intensity. The higher the ratio, the stronger the FRET signal. The YFP-apoE4-CFP-transfected cells had a much higher ratio of FRET to CFP than the YFP-apoE3-CFP-transfected cells (Fig. 3B), suggesting that FRET occurs in YFP-apoE4-CFP cells but not in YFP-apoE3-CFP cells.

Finally, we measured the change of the donor (CFP) fluorescence before and after laser photobleaching of the acceptor (YFP) fluorescence. If FRET occurs, this photobleaching will increase the donor (CFP) fluorescence because the energy cannot be transferred from CFP to YFP (66, 73, 80). After photobleaching of YFP, CFP fluorescence increased significantly (~25%) in YFP-apoE4-CFP cells (Fig. 4A), indicating the occurrence of FRET, but decreased slightly in YFP-apoE3-CFP cells, indicating the absence of FRET (Fig. 4B). The slight decrease in CFP fluorescence probably reflects photobleaching of CFP due to the partial overlap of the excitation spectra of YFP and CFP (73, 80).

**FRET in YFP-apoE4-CFP Cells Is Intramolecular**—Next, we determined if the FRET observed in YFP-apoE4-CFP cells was intramolecular, which could be caused by domain interaction, or intermolecular, which could be caused by dimerization or other protein–protein interactions. Neuro-2a cells were cotransfected with YFP-apoE4 and apoE4-CFP constructs. If intermolecular FRET occurs, we would detect FRET in cotransfected cells. After photobleaching of YFP fluorescence, CFP fluorescence did not increase in the cotransfected cells (Fig. 4C), suggesting that intermolecular FRET does not occur in these cells.

**FRET in YFP-apoE4-CFP Cells Is Caused by Domain Interaction**—To determine if the intramolecular FRET in YFP-apoE4-CFP cells was caused by domain interaction, we prepared two mutants in which domain interaction was disrupted: YFP-apoE4-Thr-61-CFP and YFP-apoE4-Ala-255-CFP, both of which have a more open structure than apoE4 (Fig. 5A). The ratios
of FRET (YFP) to CFP fluorescence were significantly lower in Neuro-2a cells expressing the mutant proteins than in cells expressing YFP-apoE4-CFP at similar levels (Fig. 5B), suggesting that the N- and the C-termini of these two apoE4 mutants are separated by more than 100 Å. Interestingly, the ratios of YFP to CFP fluorescence in cells expressing the mutant proteins were similar to those of cells expressing YFP-apoE3-CFP. Thus, the two apoE4 mutants appear to be structurally similar to apoE3, as reported (35, 36). These data strongly suggest that the FRET in YFP-apoE4-CFP cells is caused by apoE4 domain interaction.

**Fusion of Fluorescent Proteins with ApoE3 and ApoE4 Does Not Alter Their Lipid Binding Properties**—To determine whether the fusion of fluorescent proteins with apoE3 and apoE4 alter their lipid binding properties, which is one of the major functions of apoE, we measured lipid content of secreted apoE-containing lipoproteins. Both apoE4 and YFP-apoE4-CFP bound approximately 25% less phospholipid ($p < 0.05$) than apoE3 and YFP-apoE3-CFP (Fig. 6A). Low levels of cholesterol and undetectable levels of triglyceride were found in the medium (data not shown). These results indicate that fusion of fluorescent proteins with apoE3 and apoE4 does not alter their lipid binding properties, suggesting that the presence of YFP and CFP does not alter significantly the folding or structural properties of apoE. These results also indicate that the cells secreted less phospholipid with apoE4 than with apoE3, suggesting decreased phospholipid-binding capacity of apoE4. Furthermore, a strong FRET signal was detected in the medium containing YFP-apoE4-CFP (Fig. 6B), consistent with apoE4 domain interaction occurring in secreted phospholipid-rich lipoprotein particles.

**Domain Interaction Decreases Phospholipid-binding Capacity of ApoE4**—To determine whether the domain interaction is responsible for the decreased phospholipid-binding capacity of apoE4, we compared the amount of phospholipid bound by YFP-apoE4-Thr-61-CFP, in which the domain interaction had been disrupted (Fig. 5), with that bound by YFP-apoE4-CFP or YFP-apoE3-CFP. The YFP-apoE4-Thr-61-CFP bound significantly more phospholipid than YFP-
apoE4-CFP ($p < 0.05$). Importantly, YFP-apoE4-Thr-61-CFP and YFP-apoE3-CFP bound similar amounts of phospholipid (Fig. 6C). Thus, disruption of domain interaction restores phospholipid-binding capacity of apoE4 to a level similar to that of apoE3. These results strongly suggest that domain interaction decreases phospholipid-binding capacity of apoE4, leading to secretion of less phospholipid from apoE4-producing cells than from apoE3-producing cells.

In summary, the current study demonstrates that apoE4 domain interaction occurs in living neuronal cells as determined by FRET and confirms that the FRET technique is a powerful tool to detect intramolecular domain interaction of proteins in living cells. This study also reveals that domain interaction decreases phospholipid-binding capacity of apoE4, leading to decreased secretion of phospholipid from apoE4-producing cells. Consistent with this finding, human apoE–producing primary astrocytes from apoE4 knock-in mice secrete less phospholipid than those from apoE3 knock-in mice (82). Morphological and behavioral analyses of transgenic mice expressing apoE3 or apoE4 specifically in central nervous system neurons revealed significant age-dependent and excitotoxin-induced neurodegeneration and behavioral deficits in apoE4, but not apoE3, mice (55, 58). Since the structural and biophysical properties of a protein determine its physiological or pathological functions, the intraneuronal apoE4 domain interaction observed in this study might be a molecular basis for apoE4-related neurodegeneration in apoE4 transgenic mice (31, 34, 59, 60) and probably also in humans. The development of drugs that can disrupt the intraneuronal apoE4 domain interaction should be beneficial for patients with AD and possibly other neurodegenerative diseases associated with apoE4. The cell-based FRET assay developed in the current study could be used to evaluate promising drug candidates in living neuronal cells.
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FIGURE LEGENDS

Fig. 1. cDNA constructs encoding apoE3 or apoE4 fused with YFP and CFP and the use of FRET to determine apoE4 domain interaction. (A) After transient transfection of Neuro-2a cells with various apoE constructs, the intracellular apoE fusion proteins were detected by Western blotting with a monoclonal anti-FLAG. (B) Use of FRET to determine apoE4 domain interaction. N-Term, N-terminal domain; C-Term, C-terminal domain.

Fig. 2. FRET occurs in YFP-apoE4-CFP, but not in YFP-apoE3-CFP, cells as determined by imaging YFP fluorescence under excitation of CFP fluorescence. Neuro-2a cells transiently transfected with the YFP-apoE3-CFP or YFP-apoE4-CFP cDNA construct were illuminated with a Zeiss two-photon laser and examined by Zeiss confocal microscopy. To visualize CFP fluorescence, transfected cells expressing similar levels of YFP-apoE3-CFP or YFP-apoE4-CFP were excited at 458 nm and images were acquired at the emission wavelength of 465–512 nm. FRET images were acquired at the emission wavelength of YFP (543–618 nm) when CFP was excited. Representative images from one of four experiments are shown.

Fig. 3. FRET occurs in YFP-apoE4-CFP, but not in YFP-apoE3-CFP, cells as determined by measuring the ratio of FRET (YFP) fluorescence to CFP fluorescence. (A) When FRET occurs, the YFP (FRET) emission increases and the CFP emission decreases because energy is transferred from CFP to YFP. Therefore, the ratio of YFP (FRET) to CFP fluorescence can be used as a measurement of FRET intensity. (B) The ratios of FRET to CFP in Neuro-2a cells transiently expressing YFP-apoE3-CFP or YFP-apoE4-CFP at similar levels were measured by Zeiss two-photon confocal microscopy (n = 12–16 for each bar; p < 0.001).

Fig. 4. FRET occurs in YFP-apoE4-CFP, but not in YFP-apoE3-CFP or YFP-apoE4 and apoE4-CFP, cells as determined by measuring the changes of CFP fluorescence after photobleaching of YFP fluorescence. After CFP images were acquired and their fluorescence
intensities were calculated, a strong laser (514 nm, 100% power, eight 10-sec cycles) was used to photobleach YFP fluorescence in Neuro-2a cells transiently expressing YFP-apoE4-CFP (A), YFP-apoE3-CFP (B), or both YFP-apoE4 and apoE4-CFP (C). CFP images were then acquired, and their fluorescence intensities were recalculated. An increase in CFP fluorescence intensity after photobleaching of YFP fluorescence indicates the occurrence of FRET ($n = 6$ for each bar).

**Fig. 5. FRET in YFP-apoE4-CFP cells is caused by apoE4 domain interaction.** (A) Models of apoE4 domain interaction (left) and mutations that abolish domain interaction (right). (B) The ratios of FRET to CFP in Neuro-2a cells transiently expressing YFP-apoE3-CFP, YFP-apoE4-CFP, YFP-apoE4-Thr-61-CFP, or YFP-apoE4-Ala-255-CFP at similar levels were measured by Zeiss two-photon confocal microscopy ($n = 12–16$ for each bar; YFP-apoE4-CFP vs. YFP-apoE4-Thr-61-CFP or YFP-apoE4-Ala-255-CFP, $p < 0.001$).

**Fig. 6. Domain interaction decreases phospholipid-binding capacity of apoE4.** (A) The concentration of phospholipid in cell culture medium containing various forms of secreted apoE was determined before and after immunoprecipitation with anti-apoE as described under “Experimental Procedures.” The amount of phospholipid bound with apoE was calculated as the difference in the two values, normalized by protein expression levels ($n = 3$ for each bar; apoE3 vs. apoE4 or YFP-apoE3-CFP vs. YFP-apoE4-CFP, $p < 0.05$). (B) The ratios of FRET to CFP in cell culture medium containing YFP-apoE3-CFP or YFP-apoE4-CFP were measured under CFP excitation by fluorescence spectrometry ($n = 3$ for each bar; $p < 0.001$). (C) The amount of phospholipid bound with various forms of secreted apoE was determined as described in panel A. ($n = 3$ for each bar; YFP-apoE4-CFP vs. YFP-apoE3-CFP or YFP-apoE4-Thr-61-CFP, $p < 0.05$).
Xu. Q., et al, Figure 1

A

1. FLAG YFP ApoE3
2. FLAG YFP ApoE4
3. FLAG ApoE3 CFP
4. FLAG ApoE4 CFP
5. FLAG YFP ApoE3 CFP
6. FLAG YFP ApoE4 CFP

Anti-FLAG Western Blot

B

ApoE3

Excitation

Emission 490 nm

Excitation

Emission 530 nm

FRET

ApoE4

YFP

CFP

N-Term

C-Term

N-Term

C-Term

Excitation

430 nm

Excitation

430 nm

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Xu. Q., et al., Figure 2
Xu. Q., et al, Figure 3

(A) Emission intensity against wavelength (nm) showing no FRET and FRET peaks.

(B) Bar graph showing the ratio of FRET to CFP for YFP-ApoE3-CFP and YFP-ApoE4-CFP.
Xu. Q., et al, Figure 4

A  YFP-ApoE4-CFP
B  YFP-ApoE3-CFP
C  YFP-ApoE4

Pre-YFP-Bleaching  |  Post-YFP-Bleaching

CFP-Fluorescence Intensity (% of Prebleaching)

0  |  50  |  100  |  150

ApoE4-CFP

ApoE4-CFP

ApoE4-CFP
ApoE4 Mutants

Glu-255
Arg-61
Arg-112

Ala-255
Thr-61
Arg-112

ApoE4

Ratio of FRET to CFP

YFP-ApoE3-CFP
YFP-ApoE4-CFP
YFP-ApoE4-Thr-61-CFP
YFP-ApoE4-Ala-255-CFP

Xu. Q., et al, Figure 5
Xu, Q., et al, Figure 6
Apolipoprotein E4 domain interaction occurs in living neuronal cells as determined by fluorescence resonance energy transfer

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