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# HDL Mimetic Peptide 4F Mitigates Aβ-Induced Inhibition of ApoE Secretion and Lipidation in Primary Astrocytes and Microglia

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## Abstract

The apolipoprotein (apo) E ε4 allele is the primary genetic risk factor for late-onset Alzheimer's disease (AD). ApoE in the brain is produced primarily by astrocytes; once secreted from these cells, apoE binds lipids and forms high-density lipoprotein (HDL)-like particles. Accumulation of amyloid-β protein (Aβ) in the brain is a key hallmark of AD, and is thought to initiate a pathogenic cascade leading to neurodegeneration and dementia. The level and lipidation state of apoE affect Aβ aggregation and clearance pathways. Elevated levels of plasma HDL are associated with lower risk and severity of AD; the underlying mechanisms, however, have not been fully elucidated. This study was designed to investigate the impact of an HDL-mimetic peptide, 4F, on the secretion and lipidation of apoE. We found that 4F significantly increases apoE secretion and lipidation in primary human astrocytes as well as in primary mouse astrocytes and microglia. Aggregated Aβ inhibits glial apoE secretion and lipidation, causing accumulation of intracellular apoE, an effect that is counteracted by co-treatment with 4F. Pharmacological and gene editing approaches show that 4F mediates its effects partially through the secretory pathway from the endoplasmic reticulum to the Golgi apparatus and requires the lipid transporter ABCA1. We conclude that the HDL-mimetic peptide 4F promotes glial apoE secretion and lipidation and mitigates the detrimental effects of Aβ on proper cellular trafficking and functionality of apoE.

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Authors' Contributions:

DC designed and performed most of the experiments, analyzed the data, and wrote the manuscript. SOV performed Western blot analysis for experiments in primary human astrocytes. AJ assisted with Western blot analysis for apoE receptors and generated the graphical abstract. SS conducted and KK supervised the experiment for Aβ uptake by confocal microcopy and flow cytometry. GWR instructed the culture of human apoE targeted-replacement immortalized mouse astrocyte and participated in the discussion of the results. LL conceived the study, supervised the design of experiments and interpretation of results, and edited and finalized the manuscript.

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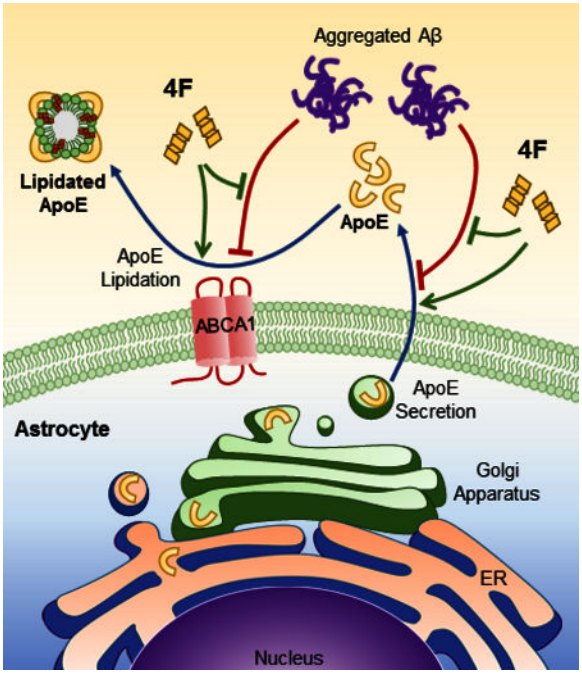
These findings suggest that treatment with such an HDL mimetic peptide may provide therapeutic benefit in AD.


## Abstract

The level and lipidation state of apoE affect the pathogenesis of Alzheimer's disease (AD). In this study, an HDL-mimetic peptide, 4F, was found to promote the secretion and lipidation of apoE and to counteract Aβ-induced inhibition of apoE secretion and lipidation in primary human astrocytes, as well as in primary mouse astrocytes and microglia. Pharmacological and gene editing approaches show that 4F mediates its effects partially through the secretory pathway from the endoplasmic reticulum (ER) to the Golgi apparatus and requires the lipid transporter ABCA1. These findings suggest the therapeutic potential of HDL mimetic peptides in AD.



### Keywords

Alzheimer's disease; apolipoprotein E; lipidation; HDL mimetic peptide; amyloid-β; astrocytes and microglia


## Introduction:

Alzheimer's disease (AD) is the leading cause of dementia in the elderly; the risk of developing AD doubles every five years after the age of 65, and over one-third of Americans over 85 are affected (Hebert et al. 2013). Although the pathogenesis of AD is not fully understood, it is widely accepted that accumulation of amyloid-β protein (Aβ) in the brain initiates a pathogenic cascade, ultimately leading to neurodegeneration and dementia (Hardy & Selkoe 2002, Mucke & Selkoe 2012). There is currently no effective therapeutic that can halt, or even slow, the progression of AD pathology.

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The apolipoprotein (apo) E ε4 allele is the strongest genetic risk factor identified to date for late-onset AD, associated with an earlier age of onset (Corder et al. 1993, Saunders et al. 1993, Strittmatter et al. 1993). In the brain, apoE is primarily secreted by astrocytes while microglia also produce apoE (Xu et al. 2006). Once secreted, apoE binds lipids and forms high-density lipoprotein (HDL)-like particles in the interstitial and cerebrospinal fluids through interactions with the ATP-binding cassette transporter A1 (ABCA1). The human apoE gene has three common alleles: ε2, ε3, and ε4 (designated apoE2, E3, and E4). The three resulting isoforms differ only at two amino acid sites in the protein. ApoE3 is the most common isoform and has a Cysteine residue at position 112 and an Arginine residue at 158. ApoE2, the rarest isoform, has Cysteine at both positions 112 and 158, whereas apoE4 has Arginine residues at both positions (Weisgraber et al. 1981).

ApoE has been extensively studied in the context of AD. It has been shown that apoE interacts with Aβ and that the level and lipidation state of apoE affect Aβ aggregation and clearance (Bu 2009, Holtzman et al. 2012, Liao et al. 2017).

ApoE4 has been shown to be poorly lipidated compared with apoE2 and apoE3 (Kanekiyo et al. 2014, Tai et al. 2014b, Hu et al. 2015, Hanson et al. 2013, Heinsinger et al. 2016), which is thought to be the cause of many of its deleterious effects (Bu 2009, Holtzman et al. 2012). Indeed, the receptor binding ability of apoE is dependent on its lipidation state; key residues involved in receptor binding become unburied when apoE is in a lipidated state (Chen et al. 2011). ApoE2, which reduces the risk for AD, has increased total levels of apoE compared to apoE3 or apoE4 (Cruchaga et al. 2012). This indicates that apoE level and lipidation state may be of crucial importance in combating the toxic Aβ pathway in AD pathogenesis.

Aggregated Aβ reduces the secretion of apoE and causes its cellular accumulation in astrocytes (LaDu et al. 2000, Handattu et al. 2013). The mechanism of this effect has not been fully described, although cAMP and β-adrenergic receptors have been shown to play a role (Igbavboa et al. 2006, Rossello et al. 2012). In addition, apoE binds to cell surface heparin sulfate proteoglycan (HSPG) and cell membrane associated receptors and competes with Aβ for uptake (Huang & Mahley 2014, Fu et al. 2016). Recent studies have also demonstrated that apoE binds to the triggering receptor expressed on myeloid cells 2 (TREM2) and regulates microglial function (Atagi et al. 2015, Bailey et al. 2015, Krasemann et al. 2017, Yeh et al. 2016). It has been shown that the LDL receptor (LDLR), but not HSPG, mediates the effects of Aβ on apoE in astrocytes (LaDu et al. 2000). This highlights the direct role of the lipid metabolism pathway in AD pathogenesis. Further, the formation of apoE/Aβ complexes is observed (Wisniewski et al. 1993, LaDu et al. 1994, Tai et al. 2014b), and may increase the ability of enzymes such as insulin degrading enzyme (IDE) to degrade Aβ in the extracellular space (Russo et al. 1998). Highly lipidated apoE promotes IDE degradation of Aβ to a greater extent than less lipidated forms (Jiang et al. 2008). Interestingly, apoE4 carriers produce less apoE/Aβ, and the complexes they do form are less stable (Tai et al. 2013). The reduced sequestration of Aβ into these complexes may hinder its degradation and lead to increased levels of toxic oligomers in the brain. However, a recent study showed that apoE minimally associates with Aβ but retains its significant influence on Aβ clearance, possibly through competing for binding to cell surface receptors (Verghese et al. 2013).

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Multiple large-scale human clinical studies have found HDL levels to be highly correlated with cognitive performance late in life, and to be inversely correlated with both AD risk and severity (Hottman et al. 2014). A number of animal studies also support the role of apoA-I/HDL in AD (Lewis et al. 2010)(Lefterov et al. 2010)(Robert et al. 2016)(Song et al. 2014). These data led to the hypothesis that apoA-I/HDL may be an attractive target for AD therapy. However, the potential of full-length apoA-I protein as a therapeutic is greatly limited by its high cost of production and lack of oral bioavailability. A series of small peptides that mimic HDL function have been developed as potential therapeutics for cardiovascular disease (Osei-Hwedieh et al. 2011, Leman et al. 2014, White et al. 2014, Getz & Reardon 2014). The most notable of these is the peptide known as 4F, which is an 18 amino acid peptide containing 4 phenylalanine (F) residues (Ac-DW**F**KA**F**YDKVAEK**F**KEA**F**-$NH_2$) (Segrest et al. 1983). 4F does not share sequence homology with any natural proteins but mimics the class A amphipathic helixes contained in HDL associated apolipoproteins such as apoA-I and apoE (Segrest et al. 1992). Animal studies have shown that treatment with 4F enhances HDL function and inhibits atherosclerosis (Navab et al. 2008). Intriguingly, animal experiments have also demonstrated that treatment with 4F reduces neuroinflammation and promotes cognitive function in atherosclerotic and AD mice (Buga et al. 2006, Handattu et al. 2009); the underlying mechanisms, however, have not been elucidated. As apoE is the primary HDL-associated protein in the brain, and 4F promotes cellular lipid efflux in the periphery (Xie et al. 2010, Liu et al. 2010, Tang et al. 2006), the beneficial effects of 4F on brain function may be mediated through apoE secretion and lipidation in the brain, which has not been explored previously.

Thus, the present study aimed to determine the impact of 4F on the secretion and lipidation of apoE in primary astrocytes and microglia, and whether 4F could mitigate the effects of Aβ therein. Our results demonstrate that 4F increases the secretion and lipidation of apoE from both mouse and human astrocytes, as well as mouse microglia, and ameliorates Aβ-induced inhibition in apoE secretion and lipidation. We also show that the 4F-mediated effects depend in part on the secretory transport pathway, from the endoplasmic reticulum (ER) to the Golgi apparatus, and the function of cell surface associated receptors. Specifically, we show that 4F-mediated enhancement of apoE secretion is abolished in the absence of ABCA1 expression. These findings suggest that 4F may reduce the detrimental effects of poorly lipidated apoE in the brain and serve as a potential therapeutic agent for AD.

## Methods:

### Primary astrocyte and microglial culture:

This study was not pre-registered. Primary glial cells were collected and cultured as previously described (Fagan et al. 1999). Briefly, neonatal pups (total n=84) of wild-type C57BL/6J mice (Stock No: 000664, The Jackson Laboratory, Bar Harbor, ME; RRID:IMSR_JAX:000664) were sacrificed within the first 3 days post-natal. Both sexes of the pups were used and no randomization was performed to allocate animals in the study. The animals were anesthetized by isoflurane inhalation prior to decapitation. Brains were

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dissected out, then cortex and hippocampal tissue were triturated into a single-cell suspension and cultured for 14 days in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 16mM HEPES buffer, 0.1mM non-essential amino acids, 2mM GlutaMAX, 2.5μg/ml amphotericin B and 50μg/ml gentamicin, at which point microglia were removed by shaking. Medium was replaced, and microglia were allowed to grow up an additional week, at which point they were shaken loose and removed again. Astrocytes were then passaged two times to increase purity. The purity of astrocyte and microglial cultures was determined by immunofluorescence analysis of markers specific for astrocytes and microglia (Fig. S1 and S2). Finally, cells were plated for treatment at $2\times10^5$ cells per well on poly-D-lysine (PDL) coated 12-well tissue culture plates and allowed to attach and grow for 2 days prior to experimental treatment. Human primary astrocytes were purchased from ScienCell (Carlsbad, CA; Cat# 1800) and cultured following the manufacturer's protocols. The peptides (with purity > 95%), 4F (Ac-DWFKAFYDKVAEKFKEAF-NH2), D-4F (same sequence as 4F but with D-amino acids), and Scrambled 4F (S. 4F) (Ac-DWFAKDYFKKAFVEEFAK-NH2), were purchased from American Peptide Company (now Bachem; Sunnyvale, CA). During treatment, cells were washed twice with sterile phosphate buffered saline (PBS) and treatments were performed in serum-free OPTI-MEM supplemented with 50μg/ml gentamicin for various durations, as defined for each experiment. No blinding was performed during the experiment. All animal procedures were prospectively reviewed and approved by the Institutional Animal Care and Use Committee (IACUC protocol # 1607–33963A) of the University of Minnesota.

### Gel electrophoresis and Western blot analysis:

After treatments were performed, media was collected and cells were lysed in ice-cold RIPA buffer. Media and cell lysates were then subjected to 12% SDS-PAGE. Proteins were transferred to PVDF membranes and probed with anti-mouse apoE (Santa Cruz Biotechnology, Dallas, TX; Cat# sc-6384; RRID:AB_634036) and tubulin (Sigma-Aldrich, St. Louis, MO; Cat# T5168; RRID:AB_477579) antibodies, followed by HRP-conjugated secondary antibody and chemiluminescence detection using Western Lighting Plus-ECL reagents (PerkimElmer, Waltham, MA; Cat# NEL103001EA). ApoE from immortalized apoE3 targeted-replacement astrocytes was probed with an anti-human apoE antibody (Millipore, Burlington, MA; Cat# 178479; RRID:AB_564230). Other primary antibodies used include: anti-glial fibrillary acidic protein (GFAP) (Millipore; Burlington, MA; Cat# MAB3402; RRID:AB_94844), anti-ionized calcium-binding adapter molecule 1 (Iba1) (Wako; Richmond, VA; Cat# 019–19741; RRID:AB_839504), anti-apoJ (clusterin) (R&D; Minneapolis, MN; Cat# AF2747; RRID:AB_2083314), anti-complement C3 (MP Biomedical; Santa Ana, CA; Cat# 0855444; RRID:AB_2334469), anti-ABCA1 (Novus; Littleton, CO; Cat# NB400–105; RRID:AB_10000630), anti-LDLR (Abnova; Taipei, Taiwan, Cat# PAB8804; RRID:AB_1676510), and anti-LRP1 (Millipore Cat# MABN1796, clone 6F8; generously provided by Dr. Guojun Bu, Mayo Clinic, Jacksonville, FL).

Non-denaturing gradient gel electrophoresis (NDGGE) was used to assess apoE lipidation. Fresh media was run on 4–20% polyacrylamide tris-glycine gels (ThermoFisher; Waltham, MA; Cat# EC6021BOX) in the absence of SDS, reducing agents or sample boiling at 125V for 5 hours. Proteins were transferred to PVDF membranes at 100V for 90 minutes and

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probed for apoE, followed by HRP-conjugated secondary antibody and chemiluminescence detection using ECL reagents. Poorly lipidated apoE was defined as complexes smaller than 8.2 nm as measured by NDGGE based on the high molecular weight marker (GE Healthcare; Buckinghamshire, UK; Cat# 17044501).

### Preparation of aggregated Aβ:

Aggregated $A\beta_{42}$ was prepared as previously described (Stine et al. 2011). Briefly, hexafluoroisopropanol (HFIP) treated $A\beta_{42}$ film, kindly provided by Dr. Mary Jo LaDu and Dr. Leon Tai (University of Illinois at Chicago), was resuspended in fresh dry DMSO, diluted in phenol-free F12 cell culture media, and incubated at 37°C for 24 hours for aggregation. Aliquots of the Aβ preparation at 0 and 24 hours were examined by LDS-NuPAGE (4–12% bis-tris gels; ThermoFisher; Waltham, MA; Cat# NP0322BOX) followed by immunoblot analysis with the anti-Aβ antibody, 6E10 (Covance (now BioLegend); San Diego, CA; Cat# SIG-39340; RRID:AB_662806), and transmission electron microscopy (TEM) as previously described (Fitz et al. 2017) (Fig. 3S). This aggregated Aβ preparation was used to treat cells.

### Pharmacological manipulation:

Primary mouse astrocytes were treated with various pharmacological agents in order to understand potential mechanisms of the effects seen. 4F was prepared in sterile PBS and used at a range of concentrations from 0.1μM to 5μM. Actinomycin D (Sigma-Aldrich; St. Louis, MO; Cat# A1410) was prepared in DMSO and used at 1μg/ml. Cycloheximide (Sigma-Aldrich; Cat# C7698) and brefeldin A (Sigma-Aldrich; Cat# B7651) were prepared in ethanol and used at 2μg/ml and 1μg/ml, respectively. Heparinase I (Sigma-Aldrich; Cat# H2519) and pronase (Sigma-Aldrich; Cat# P8811) were prepared in PBS and used at 5 units/ml and 10μg/ml, respectively.

### Targeted deletion of ABCA1 in astrocytes using CRISPR/Cas9:

Immortalized mouse astrocytes derived from human apoE3 targeted-replacement mice (Morikawa et al. 2005), generously provided by Dr. Guojun Bu (Mayo Clinic, Jacksonville, FL), were cultured in DMEM supplemented with 10% FBS, 2mM GlutaMAX, 50μg/ml gentamicin, and 10ng/ml epidermal growth factor (EGF). The cells were co-transfected with a CRISPR/Cas9 vector designed to disrupt/knock out (KO) ABCA1 gene expression, and a homology directed repair (HDR) vector, designed to incorporate genes encoding puromycin resistance as well as red fluorescence protein (RFP) into the genome in place of ABCA1. Both vectors were purchased from Santa Cruz biotechnology (Cat# sc-401086 and sc-401086-HDR, respectively). Transfected cells were visually confirmed by RFP expression, and un-transfected cells were eliminated by titration of puromycin concentration up to a final concentration of 9 μg/ml. Absence of ABCA1 protein expression was confirmed by Western blot analysis, and these cells were plated for experiments and treated with or without 4F as previously described.

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## Statistical Analysis:

Western blot results were quantified using Image J software. The amount of secreted apoE was analyzed as the ratio of apoE in medium to total apoE, where total apoE = apoE in medium + apoE in cell lysate, and expressed as relative percent in media with the amount in the vehicle treatment set as 100%. The total amount of apoE was normalized by tubulin when it was compared between different treatments. The amount of lipidated apoE was analyzed as the ratio of lipidated apoE to total apoE in medium, where total apoE = lipidated apoE + poorly lipidated apoE, and expressed as relative percent in lipidated form with the amount in the vehicle treatment set as 100%. Data were expressed as mean ± standard error (SE) from at least three independent experiments with each treatment in duplicate or triplicate. No sample size calculation was performed. Comparison of different treatments was performed by Student's t-test or analysis of variance (ANOVA) (for normally distributed data), or the Mann-Whitney rank sum test (for non-normally distributed data). SigmaPlot v13.0 (Systat Software, San Jose, CA) was used for statistical analysis. $p < 0.05$ was considered statistically significant.

## Results:

### 4F increases apoE secretion and lipidation in primary astrocytes

Due to the role of apoE in HDL-like particle formation and function, the secretion and lipidation state of apoE is highly important to its ability to perform its functions in the brain. ApoA-I has been shown to increase the secretion of apoE from peripheral macrophages (Rees et al. 1999) and primary mixed glia (Fan et al. 2011). We therefore hypothesized that the HDL-mimetic peptide 4F may mediate apoE secretion and lipidation in astrocytes. Using primary mouse astrocytes, we found that 4F induced a robust concentration- and time-dependent increase in both secretion and lipidation of apoE from these cells (Fig. 1; Fig. 2), without affecting cell survival (Fig. S4). We initially measured the levels of secreted apoE at 24 hours of treatment. We found that apoE secretion was significantly increased at 1, 2, and 5 μM 4F. At 5μM 4F, the secretion of apoE was increased to approximately 300% of control (Fig. 1A). Similarly, 4F produced a dose dependent increase in apoE lipidation as well, over 400% increase at 5μM (Fig 1B). We then used 5uM 4F to test the time-dependent effects from 2 to 24 hours, finding that both secretion and lipidation of apoE increased with time (Fig. 2). Interestingly, the 4F effect appears to begin plateauing between 6 and 12 hours of treatment (Fig. 2), although it is possible that such phenomenon was caused partly by the intrinsic saturation of the *in vitro* cell culture system or Western blotting. Despite the limitations and some variations observed, these results demonstrated that 4F treatment consistently increased apoE secretion and lipidation in these cells.

To test the specificity of 4F-mediated effects, we utilized a scrambled version of 4F. Scrambled 4F contains the same 18 amino acids as 4F, but in an altered sequence, which precludes the formation of the amphipathic alpha helical structure that is critical to the function of the peptide (Handattu et al. 2009). We found that scrambled 4F had no effect on apoE secretion or lipidation in comparison to 4F (Fig. 3A and3B), confirming the specificity of 4F-mediated effects and further indicating the necessity of the particular amphipathic structure of 4F to mediate such effects. In addition, we found that D-4F, which has the same

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amino acid sequence as 4F but is composed entirely of the D-enantiomers of each amino acid, produced the same effect as 4F on astrocyte apoE secretion and lipidation (Fig. 3C and3D). These results have significant therapeutic implications because peptides D-amino acids are less susceptible to degradation of digestive/proteolytic enzymes *in vivo*.

To determine whether the effect of 4F is selective to apoE secretion, we measured the levels of two other proteins, well known to be secreted by astrocytes, apoJ (aka clusterin) and complement C3, in the medium and cell lysate. The results showed that 4F treatment did not change the secretion of these proteins (Fig. S5), indicating the selective effect of 4F on apoE secretion. Furthermore, 4F did not affect the levels of ABCA1, LDLR, or LRP1 in treated cells (Fig. S6).

Next, considering that the regulation of apoE expression differs between mouse and human cells and that mouse apoE is structurally and functionally distinct from human apoE (Fagan et al. 1999, Zhu et al. 2012, Liao et al. 2015), we investigated whether the effects of 4F on apoE secretion and lipidation extend to human cells. To accomplish this, primary human astrocytes were cultured and treated with 4F for 6 and 24 hours, respectively, followed by apoE immunoblot analysis in the medium and cell lysate. The results showed that 4F treatment significantly increased the endogenous apoE secretion and lipidation in human astrocytes (Fig. 4), consistent with the findings in mouse astrocytes described above. These data demonstrate that 4F promotes apoE secretion and lipidation in both mouse and human astrocytes.

### 4F mitigates aggregated $A\beta_{42}$-induced inhibition of apoE secretion and lipidation

To investigate the effects of Aβ on apoE secretion and lipidation, we treated primary mouse astrocytes with different concentrations of aggregated Aβ42, followed by immunoblot analysis. We found that aggregated Aβ42 caused a dose-dependent decrease in apoE secretion, accompanied by accumulation of intracellular apoE in astrocytes (Fig. 5A), without affecting cell survival (Fig. S4). Furthermore, we found that apoE secreted from Aβ-treated astrocytes was less lipidated than that released by control cells (Fig. 5B). This indicates that Aβ not only suppresses the secretion of apoE, but also inhibits the lipidation of secreted apoE, thus potentially diminishing the function and stability of apoE as well. Intriguingly, co-treatment with 4F counteracts the inhibitory effects of Aβ on both apoE secretion and lipidation in primary mouse astrocytes (Fig. 5A and5B). The Aβ-induced inhibition of apoE secretion was also observed in primary human astrocytes, and as in mouse astrocytes, co-treatment with 4F overcame the inhibitory effect of Aβ on human apoE secretion (Fig. 5C).

In addition, microglia also produce apoE, and the importance of these cells in the context of AD is highlighted by findings that apoE interacts with TREM2 and regulates microglial function (Atagi et al. 2015, Bailey et al. 2015, Krasemann et al. 2017, Yeh et al. 2016). Therefore, we tested the effects of 4F and Aβ in primary mouse microglia. Similar results as in astrocytes were observed. 4F treatment increased, whereas Aβ treatment decreased, apoE secretion, and co-treatment with 4F mitigated the inhibitory effect of Aβ on apoE secretion in primary mouse microglia (Fig. 5D).

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### The effect of 4F on apoE secretion relies on the production of intracellular apoE but is not influenced by the process of transcription and translation *per se*

To determine whether new protein production is necessary for the 4F-inducted effects on apoE, we treated primary mouse astrocytes with actinomycin D (actD) and cycloheximide (CHX) to inhibit transcription and translation, respectively. ActD treatment alone did not affect the apoE protein levels (Fig. 6A), and the effects of 4F on apoE secretion were unaffected by the presence of actD, suggesting that pre-existing apoE mRNA was stable and sufficient to produce the new protein. In contrast, CHX treatment alone drastically reduced apoE levels in both the media and cell lysates (Fig. 6A). Interestingly, the residual amount of apoE produced in the presence of CHX was efficiently secreted into the medium with the treatment of 4F (Fig. 6A). Together, these results showed that the secretion of apoE promoted by 4F depended on the availability of intracellular apoE but the mechanism was not influenced by the processes of transcription and translation *per se*.

### 4F promotes apoE secretion in part through the protein transport pathway from the endoplasmic reticulum to the Golgi apparatus

To further study the mechanisms by which 4F enhances apoE secretion, we employed the use of brefeldin A (BFA), which interferes with the anterograde transport from the endoplasmic reticulum (ER) to the Golgi apparatus (Klausner et al. 1992). Treatment of primary mouse astrocytes with BFA drastically inhibited basal apoE secretion. Further, BFA treatment significantly reduced the 4F-induced effect on apoE secretion (Fig. 6B). Interestingly, in the presence of BFA, 4F treatment showed a trend increase in apoE secretion compared to BFA alone, although this effect did not reach statistical significance (Fig. 6B). This indicates that there might be a delay in the effects of BFA on the secretory pathway during the co-treatment with 4F or there might be other pathways involved in mediating the effects of 4F on apoE secretion.

### 4F-stimulated apoE secretion does not involve heparin sulfate proteoglycan but requires cell surface receptors

ApoE is known to bind to heparin sulfate proteoglycan (HSPG) and other receptors on the cell surface to produce a surface-bound pool of apoE, which can be released into the media (Huang & Mahley 2014). Therefore, we aimed to determine if 4F-stimulated apoE secretion involves the release of this extracellular matrix or receptor-bound pool of apoE. To cleave cell surface HSPG and receptors, primary mouse astrocytes were pre-treated with heparinase or pronase for 1 hour, respectively. Following a thorough wash to remove apoE released by this treatment, the cells were treated with 4F or vehicle for 2 hours in the absence of the proteolytic agents. We found that heparinase treatment had no effects on 4F-stimulated apoE secretion. 4F produced an equally significant increase in apoE secretion after heparinase treatment (Fig. 6C), indicating that 4F does not induce its effect by releasing HSPG-bound pools of apoE. Interestingly, while pronase treatment did not affect the basal level of apoE secretion, it abolished 4F-stimulated apoE secretion (Fig. 6C). Together, these results show that 4F-stimulated apoE secretion does not involve cell surface HSPG but relies on the presence of other cell surface receptors.

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### 4F-mediated apoE secretion requires ABCA1

The inhibition of 4F-mediated apoE secretion by pronase treatment led us to question which specific cell-surface protein(s) are required for this phenomenon to occur. ABCA1 is the primary cell surface receptor responsible for the transfer of lipids onto nascent HDL particles (Oram & Heinecke 2005). As such, we aimed to determine whether ABCA1 is required for the 4F-mediated effects on apoE to occur. We utilized immortalized mouse astrocytes with a targeted replacement of mouse apoE for human apoE3 (Morikawa et al. 2005). Using commercially available vectors based on the CRISPR/Cas9 technology (Santa Cruz biotechnology), the ABCA1 gene was disrupted/knocked out in these cells. Transfection and loss of ABCA1 protein expression were confirmed by fluorescent imaging and Western blot analysis (Fig. 7A and7B). We found that total levels (secreted and cellular) of apoE were markedly reduced in ABCA1 KO cells (Fig. 7B). This finding indicates that cellular production of apoE is downregulated in the absence of ABCA1, consistent with findings in animal models (Hirsch-Reinshagen et al. 2005, Koldamova et al. 2005, Wahrle et al. 2005). Compared with ABCA1-intact wild type (WT) astrocytes, 4F failed to induce apoE secretion in ABCA1 KO cells (Fig. 7B). Importantly, 4F-mediated enhancement of apoE secretion could be detected under the condition of reduced cellular apoE levels, as observed in our experiments using the translational inhibitor CHX (Fig. 6A). Thus, the results indicate that the presence of ABCA1 is required for 4F-mediated effects on apoE secretion.

## Discussion:

In the current study, we described the ability of 4F, an 18 amino acid HDL-mimetic peptide, to increase the secretion and lipidation of apoE in primary human astrocytes as well as primary mouse astrocytes and microglia. The primary functions of apoE require it to be part of an HDL-like particle in the brain, and mounting evidence suggests that many of its critical roles are influenced by the degree to which apoE is lipidated. ApoE4, the primary genetic risk factor for late onset AD, has been shown to be poorly lipidated compared with apoE2 and apoE3 (Kanekiyo et al. 2014, Tai et al. 2014b, Hu et al. 2015, Hanson et al. 2013, Heinsinger et al. 2016). As the lipidation status of apoE affects its function and receptor binding capacity (Bu 2009, Koldamova et al. 2014), deficits in lipidation may underlie the pathogenic effects of apoE4. Therefore, increasing the lipidation of apoE may be a viable therapeutic avenue for AD treatment.

A number of studies have shown that oligomeric species of Aβ correlate better with cognitive impairment than Aβ plaque burden in AD (Haass & Selkoe 2007, Lesne et al. 2013, Selkoe & Hardy 2016). Aβ has also been previously shown to inhibit apoE secretion from astrocytes (LaDu et al. 2000, Igbavboa et al. 2006, Handattu et al. 2013). Our results corroborate these previous findings on the inhibition of apoE secretion by Aβ in both mouse and human astrocytes as well as in mouse microglia, and further demonstrate that lipidation of secreted apoE from primary mouse astrocytes is also inhibited by Aβ, which may reduce apoE function, stability, and receptor binding capacity. The interaction between Aβ and apoE may be a crucial part of early pathogenesis, in which early Aβ insults reduce apoE secretion and lipidation, leading to reduced Aβ clearance and thus elevated levels of

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aggregated Aβ in the brain. This in turn may create a vicious feed-forward cycle in which these elevated levels of aggregated Aβ cause a further decline in apoE secretion and lipidation. Blocking this circular pathway with a pharmacological agent that increases apoE secretion and lipidation may slow or even halt the progression of AD. Interestingly, Verghese and colleagues showed that the association between apoE and Aβ was minimal in the cerebrospinal fluids in human apoE targeted-replacement mice and that apoE-deficient astrocytes cleared Aβ from the medium more efficiently than apoE-expressing astrocytes (Verghese et al. 2013). They further showed that apoE and Aβ compete for the same LRP1-mediated clearance pathway, suggesting that reducing apoE levels would enhance Aβ clearance (Verghese et al. 2013). We examined the effect of co-treatment of 4F on Aβ uptake in primary mouse astrocytes by confocal microscopy and flow cytometry as described previously (Omtri et al. 2012). The results showed that 4F did not interfere with Aβ uptake (Fig. S7). Importantly, chronic treatment with 4F resulted in a significant reduction of amyloid deposition in the APP/PS1 model of AD (Handattu et al. 2009), indicating that 4F may facilitate brain Aβ clearance through multiple mechanisms *in vivo*.

When the effect of Aβ on reducing secretion of apoE was described initially, it was postulated that the Aβ-induced effect might be protective, based on the assumption that an increase in the cellular production of apoE counteracts the detrimental effects of Aβ (LaDu et al. 2000, Igbavboa et al. 2006). However, more recent studies indicate that apoE must be secreted and properly lipidated in order to perform its canonical functions, including lipid/cholesterol transport, synapse regeneration, immune modulation, and clearance/degradation of Aβ (Kanekiyo et al. 2014, Tai et al. 2014b, Hu et al. 2015, Hanson et al. 2013). As such, Aβ-induced accumulation of intracellular apoE and simultaneous inhibition of secretion is considered detrimental. We have found that co-treatment of astrocytes with 4F mitigates the Aβ-induced inhibitory effects on apoE secretion and lipidation. In the presence of 4F, astrocytes exposed to Aβ secrete similar amounts of apoE as control cells, and the secreted apoE is lipidated as efficiently as in the controls. Consistent with our findings, it has been shown that a hybrid apoE/apoA-I-mimetic peptide, Ac-hE18A-NH2, mitigated the inhibitory effect of Aβ in the U251 astrocyte cell line (Handattu et al. 2013). These results suggest that 4F or related apo mimetic peptides can counteract the detrimental effects of Aβ on astrocytes, potentially serving as a protective agent against AD. These findings *in vitro* have significant implications *in vivo*. Although it remains a topic of debate whether an increase or decrease in total apoE levels is protective against AD, improving the lipidation state of apoE ameliorates cognitive deficits in the presence or absence of amyloid pathology regardless of apoE genotype (Cramer et al. 2012, Fitz et al. 2013, Boehm-Cagan & Michaelson 2014).

In the present study, we showed that 4F treatment consistently produced a robust increase in apoE secretion and lipidation in astrocytes throughout the experiments. The mechanisms by which 4F promotes apoE secretion were explored with the use of pharmacological agents and CRISPR/Cas9-mediated gene editing approaches. The effect of 4F occurs in part through the classical ER-Golgi secretion pathway, as inhibition of this pathway by BFA partially blocks the 4F-induced enhancement of apoE secretion. Our experiments with heparinase demonstrate that cell surface HSPG-associated apoE does not contribute to the effect of 4F on apoE secretion, which is in line with a previous study showing that the Aβ-induced elevation of intracellular apoE does not depend on interactions with HSPG (LaDu et

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al. 2000). However, removal of other cell surface receptors with pronase abolishes the increase of apoE secretion by 4F in primary astrocytes. Furthermore, using ABCA1 KO astrocytes generated by using CRISPR/Cas9 technology, we show that the effect of 4F on apoE secretion requires the presence of ABCA1. Notably, it was reported recently that an apoE-mimetic peptide ameliorated apoE4-driven cognitive and brain pathologies through activation of ABCA1 (Boehm-Cagan et al. 2016). These findings corroborate the importance of ABCA1 in apoE-targeted therapeutic development.

Highlighting the importance of ABCA1 in the context of AD, several studies have shown that ABCA1 deficiency exacerbates amyloid pathology in AD mice (Hirsch-Reinshagen et al. 2005, Koldamova et al. 2005, Wahrle et al. 2005). ABCA1 KO mice exhibit drastically reduced apoE levels and lipidation. In contrast, apoE KO AD mice have reduced amyloid deposition (Bales et al. 1997, Irizarry et al. 2000) and tau pathology (Shi et al. 2017). Furthermore, immunotherapy against apoE was associated with reduced Aβ deposition in AD mice (Kim et al. 2012), an effect that has recently been recapitulated using an antibody targeting only non-lipidated forms of apoE (Liao et al. 2018). These findings, together, indicate that the lipidation state of apoE may influence AD pathology more so than the absolute level of apoE.

In line with these findings, ABCA1 overexpression is associated with elevated apoE levels and lipidation, and attenuates AD pathology (Wahrle et al. 2008). Bexarotene and other nuclear receptor agonists, which upregulate genes including ABCA1, have been shown to increase apoE level and lipidation, while reducing amyloid pathology in mice (Cramer et al. 2012, Zelcer et al. 2007, Skerrett et al. 2014, Donkin et al. 2010, Corona et al. 2016), although the effect on amyloid pathology was not observed in all studies (Veeraraghavalu et al. 2013, Tesseur et al. 2013). Importantly, these agents produce deleterious peripheral effects on triglyceride production and liver health (Hong & Tontonoz 2014, Tousi 2015, Tai et al. 2014a), as well as off-target effects inherent when targeting promiscuous transcription factors, indicating that alternative methods of increasing apoE lipidation may provide safer and greater benefits to those suffering from AD.

The potential for development of full-length HDL-associated apolipoproteins such as apoA-I or apoE as therapeutics is limited by their size, structure and post-translational modifications, making small HDL-mimetic peptides, such as 4F, attractive candidates in this regard. Notably, unlike other HDL-mimetic peptides (Leman et al. 2014, White et al. 2014), 4F has been tested in three human clinical trials with 50, 152 and 62 individuals, respectively, for cardiovascular disease; 4F was found to be safe and well-tolerated when administered orally or by injections, and improved HDL anti-inflammatory properties (Bloedon et al. 2008, Watson et al. 2011, Dunbar et al. 2017). Further, our finding that D-4F improves apoE secretion and lipidation to the same extent as 4F has significant implications for therapeutic development, because D-4F is orally bioavailable with a longer half-life than L-4F *in vivo* (Navab et al. 2005).

Our studies indicate that the small peptide, 4F, may harbor the beneficial effects of apoA-I/HDL in a form that is both more economically viable and much easier to deliver to the brain. Notably, D-4F, in the presence of a low/ineffective dose of a statin drug, has been

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shown to mitigate memory deficits and amyloid pathology in the APP/PS1 model of AD (Handattu et al. 2009). In that study, D-4F with pravastatin was administered orally in drinking water to 4–5 month old male APP/PS1 mice for 3 months, using scrambled D-4F with pravastatin and drinking water alone as controls, followed by behavioral assessment and biochemical analyses. The results showed that treatment with D-4F+pravastatin improved the learning and memory performance of APP/PS1 mice in the Morris water maze test and led to a >50% reduction in Aβ load compared with the controls (Handattu et al. 2009). Further analysis showed that D-4F+pravastatin treatment did not alter APP expression or proteolytic processing but was associated with a decrease in glial activation and inflammatory markers in the brain. Thus, it was concluded that D-4F+pravastatin treatment inhibits Aβ deposition and improves cognitive function through exerting anti-inflammatory actions in the brain (Handattu et al. 2009). These findings strongly support the beneficial effects of 4F treatment *in vivo*, although a study with 4F in the absence of any statin drug will be needed for confirmation. In addition, the role of apoE was not investigated in that study. As apoE plays a pivotal role in Aβ metabolism and immune modulation, and based on the findings in the present study, we hypothesize that the enhancement of apoE secretion and lipidation may underlie the beneficial effects of 4F treatment in AD mice. Further studies will be required to test this hypothesis.

Targeting apoE is a promising approach for AD therapy. However, simply altering the level of apoE may not be sufficient, particularly in individuals carrying the apoE4 allele. Mounting evidence shows that the lipidation status of apoE dictates its function and relative contribution to AD risk. The present study has demonstrated that the HDL-mimetic peptide, 4F, promotes secretion and lipidation of apoE from astrocytes and microglia, in both the presence and absence of Aβ, suggesting that HDL-mimetic peptides such as 4F may serve as effective apoE-modulating agents against AD.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations used:

| | |
|---|---|
| **ActD** | actinomycin D |
| **AD** | Alzheimer’s disease |

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| | |
|---|---|
| **Aβ** | amyloid-β |
| **ANOVA** | analysis of variance |
| **apoA-I** | apolipoprotein A-I |
| **apoE** | apolipoprotein E |
| **apoJ** | apolipoprotein J |
| **ABCA1** | ATP-binding cassette transporter A1 |
| **BFA** | brefeldin A |
| **C3** | complement component 3 |
| **CAA** | cerebral amyloid angiopathy |
| **CHX** | cycloheximide |
| **DAPI** | 4’,6- diamidino-2-phenylindole |
| **DMEM** | Dulbecco’s Modified Eagle Medium |
| **EGF** | epidermal growth factor |
| **ER** | endoplasmic reticulum |
| **FITC** | fluorescein isothiocyanate |
| **GFAP** | glial fibrillary acidic protein |
| **HDL** | high-density lipoprotein |
| **HDR** | homology directed repair |
| **Hep** | heparinase I |
| **HRP** | horseradish peroxidase |
| **HSPG** | heparin sulfate proteoglycan |
| **Hep** | heparinase I |
| **Iba1** | ionized calcium- binding adapter molecule 1 |
| **IDE** | insulin degrading enzyme |
| **KO** | knock out |
| **NDGGE** | non-denaturing gradient gel electrophoresis |
| **LDLR** | low density lipoprotein receptor |
| **LPS** | lipopolysaccharide |
| **LRP1** | low density lipoprotein receptor-related protein 1 |

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| | |
|---|---|
| **MFI** | mean fluorescence intensity |
| **PBS** | phosphate buffered saline |
| **PDL** | poly-D-lysine |
| **pro** | pronase E |
| **RFP** | red fluorescent protein |
| **SDS-PAGE** | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| **TEM** | transmission electron microscopy |
| **TR** | targeted replacement |
| **TxRed** | Texas Red |
| **Veh** | vehicle |
| **WT** | wild-type |

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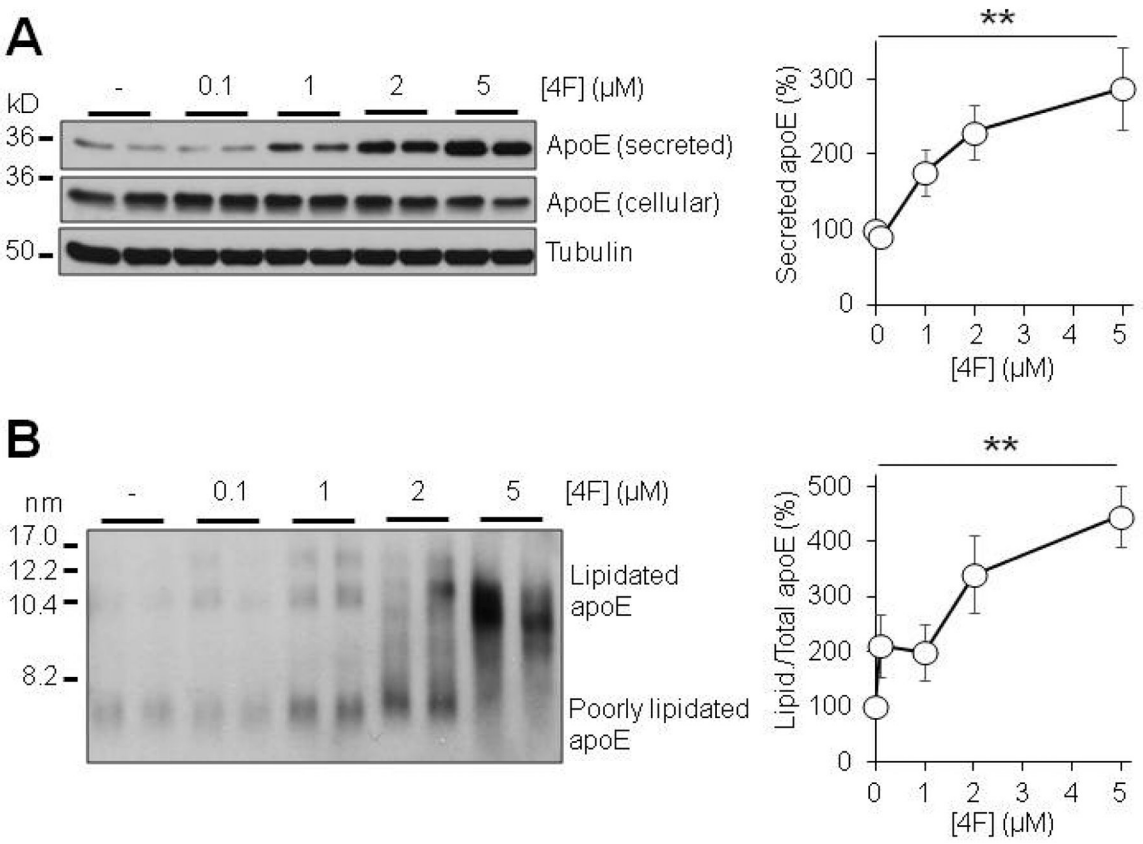


**Figure 1:**

*Dose dependent effects of 4F on apoE secretion and lipidation*. Primary mouse astrocytes were treated for 24 hours with 0–5μM 4F in serum-free OPTI-MEM. (A) SDS-PAGE and (B) NDGGE were performed to determine the relative secretion and lipidation state of apoE, respectively. Tubulin was used as a loading control. Results were obtained from n=3 independent experiments with each treatment in duplicate. ** = $p < 0.01$.

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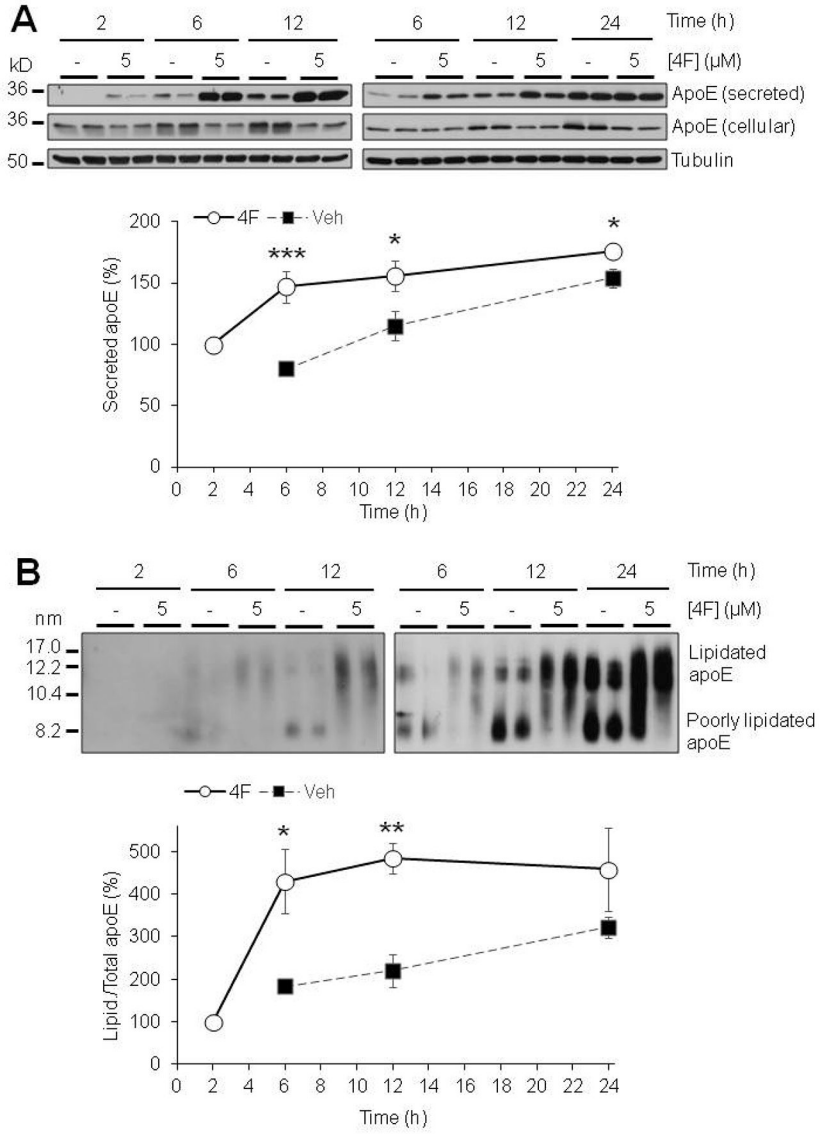


**Figure 2:**

*Time course for the effects of 4F on apoE secretion and lipidation.* Primary mouse astrocytes were treated for 2–24 hours with 5μM 4F in serum-free OPTI-MEM. (A) SDS-PAGE and (B) NDGGE were performed on samples to determine apoE secretion and lipidation. Tubulin was used as a loading control. As the level of secreted/lipidated apoE at Veh-2 h was not detectable, the level at 4F-2 h was set as 100%. Results were obtained from n=2–4 independent experiments with each treatment in duplicate. * = $p$ <0.05, ** = $p$ <0.01, *** = $p$ < 0.001.

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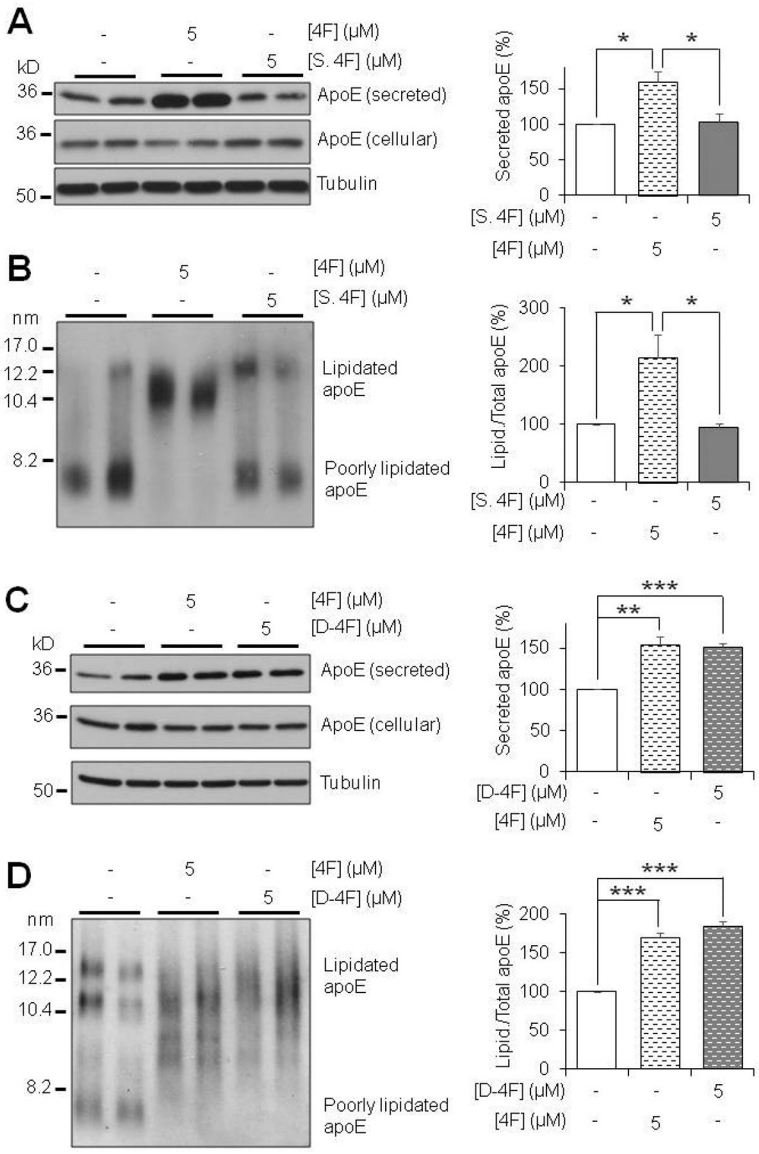


**Figure 3:**

*Scrambled 4F has no effect on apoE Secretion or Lipidation, while D-4F is equally effective as 4F.* (A,B) Primary mouse astrocytes were treated for 6 hours with 5μM 4F or Scrambled 4F (S. 4F) in serum-free OPTI-MEM. (A) SDS-PAGE and (B) NDGGE were performed to determine the relative secretion and lipidation state of apoE, respectively. (C,D) Primary mouse astrocytes were treated for 6 hours with 5 μM 4F or D-4F in serum-free OPTI-MEM. (C) SDS-PAGE and (D) NDGGE were performed to determine the relative secretion and lipidation state of apoE, respectively. Tubulin was used as a loading control. Results were obtained from n=3 independent experiments with each treatment in duplicate. * = $p$ <0.05, ** = $p$ < 0.01, *** = $p$ < 0.001.

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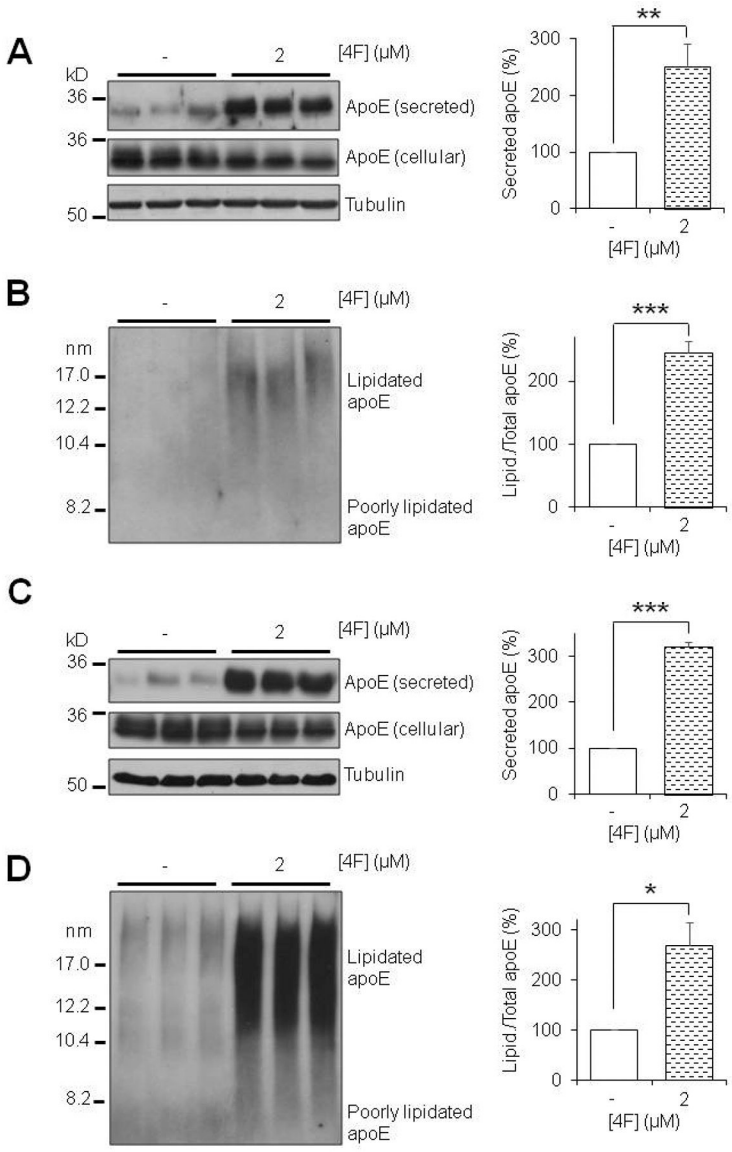


**Figure 4:**

*4F enhances apoE secretion and lipidation in primary human astrocytes.* Primary human astrocytes were treated for 6 hours (A, B) or 24 hours (C,D) with 5μM 4F in serum-free OPTI-MEM. (A,C) SDS-PAGE and (B,D) NDGGE were performed to determine the relative secretion and lipidation state of apoE, respectively. Tubulin was used as a loading control. Results were obtained from n=3 independent experiments with each treatment in triplicate. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

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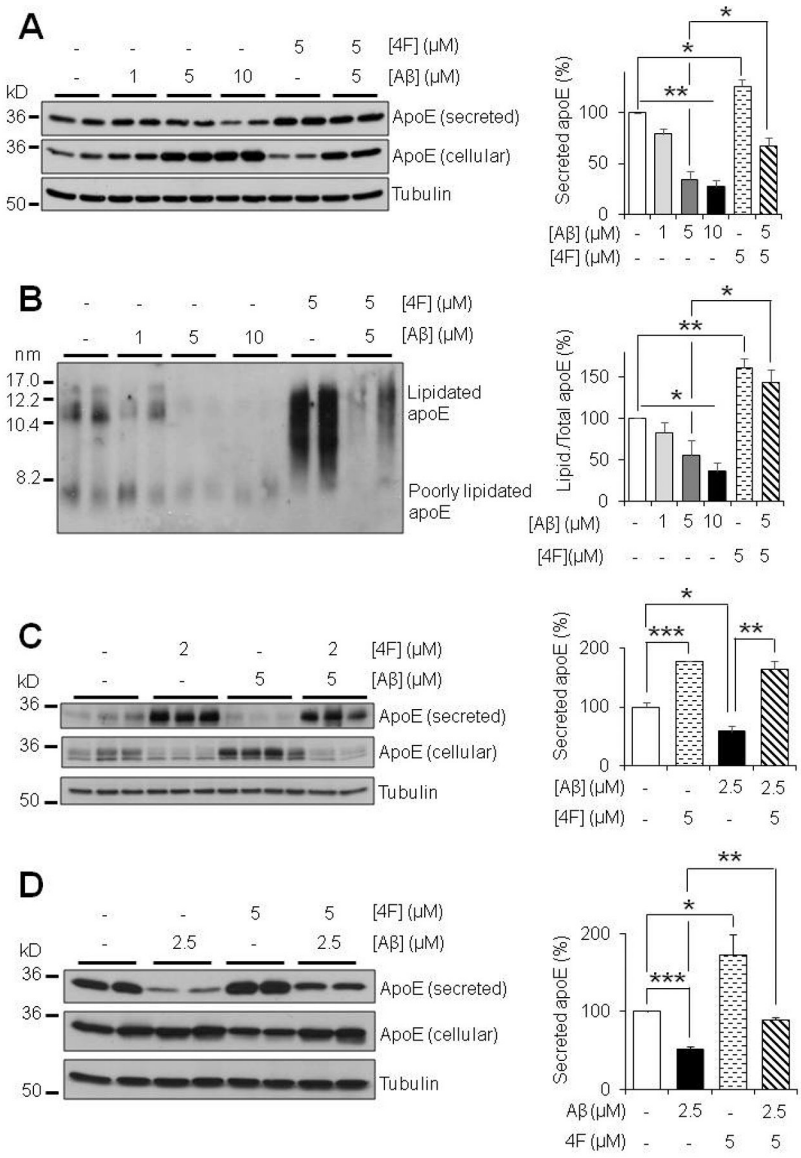


**Figure 5:**

*Aggregated Aβ inhibits apoE secretion and lipidation and 4F counteracts the inhibitory effects of Aβ in astrocytes and microglia*. (A,B) Primary mouse astrocytes were cultured for 20 hours with 1–10 μM $A\beta_{42}$ aggregates in absence or presence of 5 μM 4F in serum-free OPTI-MEM. (C) Primary human astrocytes were treated for 24 hours with 5 μM $A\beta_{42}$ aggregates and/or 2 μM 4F in serum-free OPTI-MEM. (D) Primary mouse microglia were treated for 24 hours with 2.5 μM $A\beta_{42}$ aggregates and/or 5 μM 4F. (A, C, D) SDS-PAGE and (B) NDGGE were performed to determine the relative secretion and lipidation state of apoE, respectively. Tubulin was used as a loading control. Results were obtained from n=3 independent experiments with each treatment in duplicate or triplicate. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

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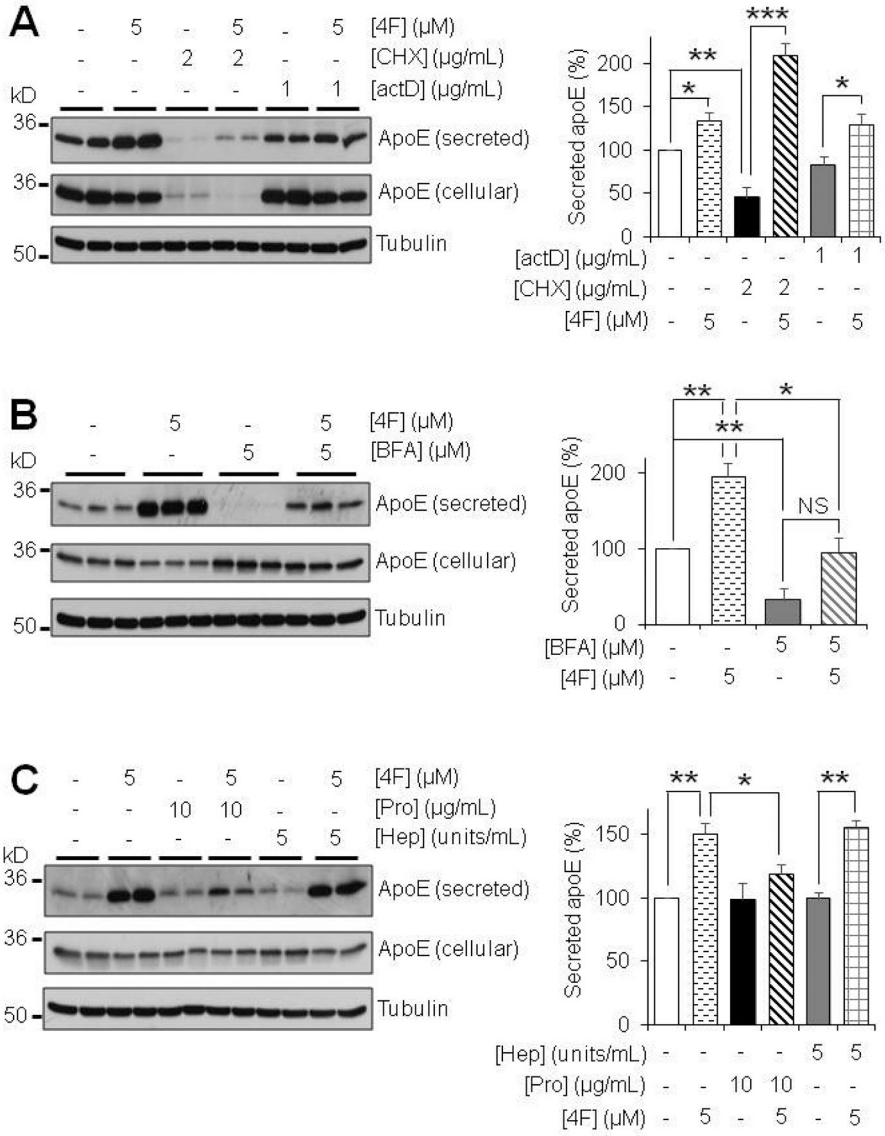


**Figure 6:**

*4F depends on protein production, partly the canonical secretory pathway, and cell-surface receptors for its effect in primary mouse astrocytes.* (A) Effects of protein transcription and translation inhibition on apoE secretion. Primary mouse astrocytes were treated for 12 hours with 1 μg/mL actinomycin D (actD) or 2 μg/mL cycloheximide (CHX) in serum-free OPTI-MEM with or without 4F. (B) Effect of inhibiting the protein secretory pathway on apoE secretion. Primary mouse astrocytes were treated for 2 hours with 5 μM Brefeldin A (BFA) in serum-free OPTI-MEM in the presence or absence of 5 μM 4F. (C) Effects of degrading heparin sulfate proteoglycan or all cell surface proteins on apoE secretion. Primary mouse astrocytes were pre-treated for 1 hour with 5 units/mL heparinase I (Hep) or 10 μg/mL Pronase E (Pro) in serum-free OPTI-MEM, respectively. Cells were then washed twice with PBS and treated with 5μM 4F for 2 hours. SDS-PAGE was performed on samples to determine the relative amount of apoE secreted. Tubulin was used as a loading control.

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Results were obtained from n=3 independent experiments with each treatment in duplicate. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$; NS, not significant ($p = 0.052$).

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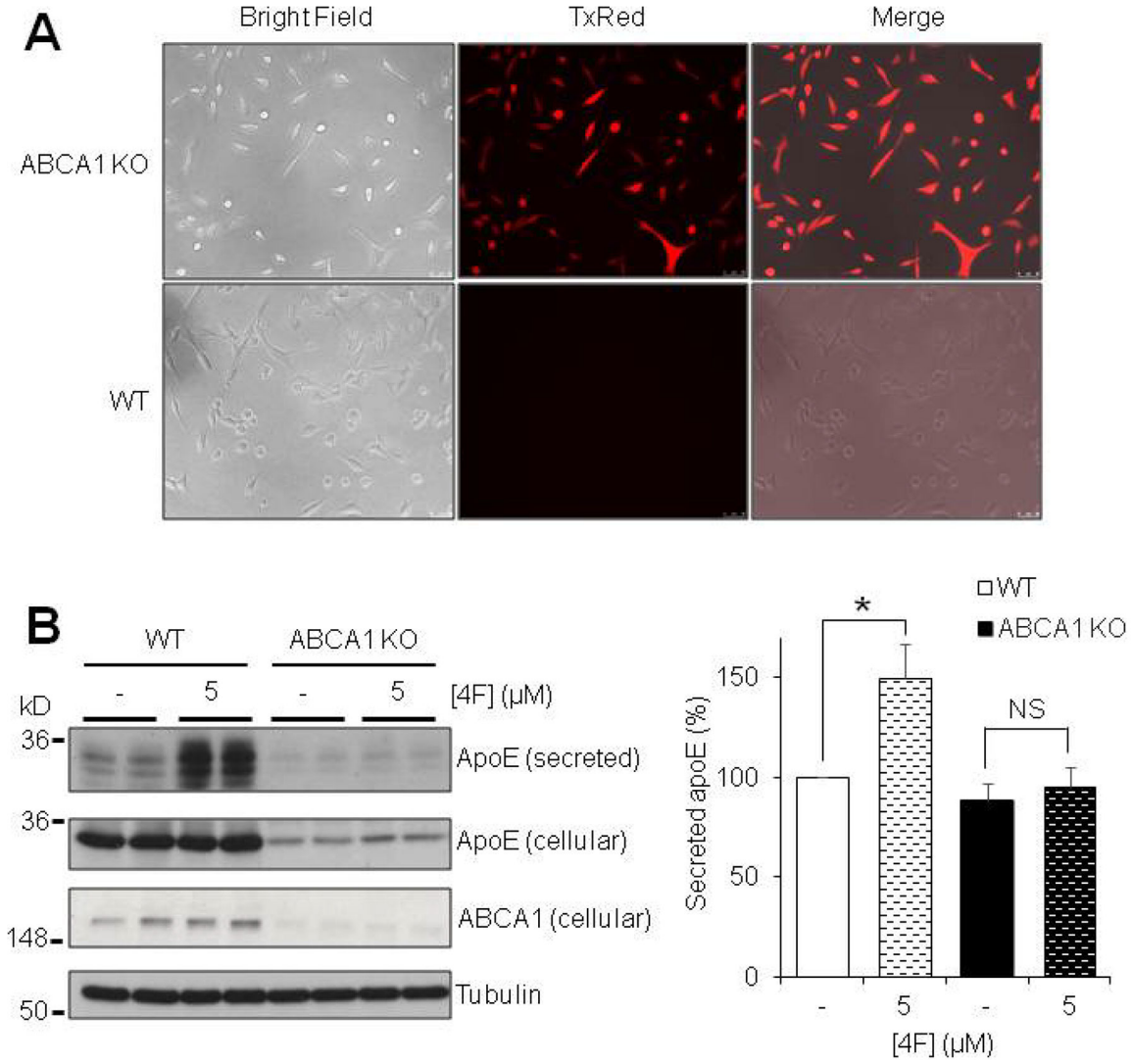


**Figure 7:**

*4F-induced elevation of apoE secretion requires ABCA1.* Immortalized apoE3 TR astrocytes were transfected with an ABCA1-specific CRISPR/Cas9 vector and a homology directed repair (HDR) vector for puromycin resistance and red fluorescence protein (RFP) to establish a stably transfected ABCA1 KO cell line. (A) Transfection is confirmed by continuous expression of RFP. Un-transfected apoE3 TR cells (WT, normal ABCA1 expression) show no RFP signal. (B) ABCA1 WT and ABCA1 KO apoE3 TR astrocytes were treated for 6 hours with or without 5μM 4F in serum-free OPTI-MEM. SDS-PAGE was performed on samples to determine the relative amount of apoE secreted, as well as ABCA1 protein expression levels. Tubulin was used as a loading control. Results were obtained from n=3 independent experiments with each treatment in duplicate. * = $p < 0.05$; NS, not significant.