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Haploinsufficiency of human APOE reduces amyloid deposition in a mouse model of Aβ amyloidosis

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Abstract

Apolipoprotein E ε4 (*APOE* ε4) is the strongest genetic risk factor for Alzheimer's disease (AD). Evidence suggests that the effect of apoE isoforms on amyloid-β (Aβ) accumulation in the brain plays a critical role in AD pathogenesis. Like in humans, apoE4 expression in animal models that develop Aβ-amyloidosis results in greater Aβ and amyloid deposition than with apoE3 expression. However, whether decreasing levels of apoE3 or apoE4 would promote or attenuate Aβ-related pathology has not been directly addressed. To determine the effect of decreasing human apoE levels on Aβ accumulation *in vivo*, we generated human *APOE* isoform haploinsufficient mouse models by crossing *APPPS1*-21 mice with *APOE* isoform knock-in mice. By genetically manipulating *APOE* gene dosage, we demonstrate that decreasing human apoE levels, regardless of isoform status, results in significantly decreased amyloid plaque deposition and microglial activation. This differences in amyloid load between apoE3 and apoE4 expressing mice were not due to apoE4 protein being present at lower levels than apoE3. These data suggest that current therapeutic strategies to increase apoE levels without altering its lipidation state may actually worsen Aβ amyloidosis, while increasing apoE degradation or inhibiting its synthesis may be a more effective treatment approach.

Keywords

Apolipoprotein E; Haploinsufficiency; Aβ; Amyloid; Plaque; Alzheimer's disease

Introduction

Aggregation and accumulation of amyloid β (Aβ) in the brain is a key initiating factor in the pathogenesis of Alzheimer's disease (AD) (Holtzman et al., 2011). Familial AD-causing mutations in *APP*, presenilin 1 (*PS1*), and *PS2* genes have provided strong support for the crucial role of Aβ aggregation in AD pathogenesis (Holtzman et al., 2011). The strongest genetic risk factor for the most common form of late-onset AD is the ε4 allele of the apolipoprotein E (*APOE*) gene (Strittmatter et al., 1993). Although several mechanisms for the effect of apoE4 on AD pathogenesis have been proposed, the primary pathway appears to be the differential effects of apoE3 and apoE4 isoforms on Aβ aggregation and clearance (Kim et al., 2009a; Castellano et al., 2011).

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In addition to isoform status, it appears that the amount of apoE also plays a role in determining the extent of Aβ accumulation in the brain. There has been intense debate as to whether potential AD therapeutics should increase or decrease apoE levels (Ashford, 2002; Rebeck et al., 2002; Teter et al., 2002; Osherovich, 2009). Initial studies clearly indicated that the deletion of the endogenous murine *Apoe* gene causes a dramatic decrease in fibrillar and total Aβ deposition in APP transgenic mouse models (Bales et al., 1997). However, whether decreasing human apoE levels will have the same beneficial effect or not has not yet been answered. Some recent studies indirectly suggest that increasing, rather than decreasing, human apoE levels may be a good therapeutic approach. Therefore, understanding how modulation of human apoE levels will alter AD pathology must be addressed prior to the development of apoE targeting drugs.

To directly test the effect of human *APOE* gene dosage on amyloid pathology, we generated Aβ depositing mouse models homozygous or hemizygous for the *APOE3* or *APOE4* gene. *APPPS1-21* mice with two copies of *APOE* (*APOE3/3* or *APOE4/4*) or one copy of *APOE* (APOE3/− or APOE4/−) were generated by breeding *APPPS1*-21 mice with human *APOE* knock-in mice. Compared with the respective homozygous mice, APOE3/− and APOE4/− hemizygous mice had significantly decreased Aβ accumulation, amyloid deposition, and microgliosis. Our results strongly suggest that the reduction of human apoE levels, regardless of the isoform present, will decrease Aβ-related AD pathology.

Materials and Methods

Generation of human APOE isoform mice with APPswe/PS1(L166P) mutant transgenes

To determine the gene dosage effect of human *APOE3* and *APOE4* on amyloid deposition, we used knock-in mouse models in which the endogenous murine *Apoe* gene is replaced with the *APOE3* or *APOE4* gene. Breeding pairs were gifts from Dr. Patrick Sullivan (Sullivan et al., 1997). *APPPS1*-21 mice overexpress a human *APP* with a Swedish mutation (KM670/671NL) and mutant *PS1* with the L166P mutation. Breeding pairs were obtained from Dr. Mathias Jucker (Radde et al., 2006). To replace the murine *Apoe* gene with human *APOE* isoforms, *APPPS1*-21 mice were bred with either *APOE3/3* or *APOE4/4* knock-in mice. *APPPS1*-21/*APOE3*/*Apoe* mice and *APOE3/Apoe* mice from the first generation were bred with each other to generate *APPPS1*-21/*APOE3/3* and *APOE3/3* mice. *APPPS1*-21/*APOE3/3* and *APOE3/3* mice were then bred to generate more *APPPS1*-21/*APOE3/3* mice. After successful generation of *APPPS1*-21/*APOE3/3* mice at the third generation, *APPPS1*-21/*APOE3/3* mice were bred with *Apoe* knockout mice (Jackson Laboratory). *APPPS1*-21/*APOE3/*− mice from the fourth generation were bred with *APOE3/3* mice to generate *APPPS1*-21/*APOE3/3* and *APPPS1*-21/*APOE3/*− mice. Littermates generated at the fifth generation were utilized in our analyses. Similarly, *APPPS1*-21/*APOE4/4* and *APPPS1*-21/*APOE4/*− mice were generated by using the same mating strategy. All mice used in this study were maintained on a C57BL/6J background.

Quantitative real-time PCR (qPCR)

mRNAs were extracted from frozen cortical tissue using Trizol and reverse transcribed with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). qPCR was performed in ABI 7500 using the default thermal cycling condition with Power SYBR (Applied Biosystems) with the following primers; forward primer: TTGCTGGTCACATTCCTGG and reverse primer: AGGTAATCCCAAAAGCGACC. Mouse GAPDH endogenous control was used as a normalization reference. Relative mRNA levels were calculated by comparative Ct method. To confirm the specificity of qPCR reactions, dissociation curves were analyzed at the end of the qPCR assay.

Western blots

Cortical tissues were gently lysed in PBS and modified RIPA (1% NP-40, 1% sodium deoxycholate, 25mM Tris-HCl, 150mM NaCl) in the presence of 1x protease inhibitor mixture (Roche, Indianapolis, IN). Tissue homogenates were centrifuged at 18,000 rcf for 30 min. Equal amounts of protein for each sample were run on 4–12% Bis-Tris XT gels (Bio-Rad, Hercules, CA) and transferred to PVDF membranes. Blots were probed with the following antibodies; apoE (Academy Bio-medical, Houston, TX), APP (ZYMED, Carlsbad, CA), PS1-NTF (EMD Chemicals, Germany), BACE1 (Cell Signaling, Beverly, MA), SYP (Sigma, St. Louis, MO), GluR2/3/4 (Cell Signaling), NMDAR2b (Cell Signaling), PSD95 (Millipore, Billerica, MA) and tubulin (Sigma). Tubulin-normalized band intensity was quantified using NIH ImageJ software.

Sandwich enzyme-linked immunosorbent assay (ELISA) for Aβ and apoE

Cortical tissues were sequentially homogenized with PBS, modified RIPA, and 5M guanidine HCl buffer. Tissue homogenates were centrifuged at 18,000 rcf for 30 min after each extraction. The levels of Aβ and apoE were measured by ELISA. For Aβ ELISA, HJ2 (anti-Aβ35-40) and HJ7.4 (anti-Aβ37-42) were used as capture antibodies and HJ5.1-biotin (anti-Aβ13-28) as the detection antibody. The anti-apoE monoclonal antibody WUE4 and another anti-apoE antibody (Calbiochem, San Diego, CA) were used for apoE ELISA.

Histology

Histology, staining, immunohistochemistry, and quantitative analysis were performed as described except that we utilized biotinylated mouse monoclonal antibody HJ3.4, 1:1000, targeted against amino acids 1–13 of the human Aβ sequence) to detect Aβ in tissue sections (Kim et al., 2009b).

Statistical analysis

To determine the statistical significance (*$p<0.05$, **$p<0.01$, ***$p<0.001$), we first tested whether our data sets passed the equal variance test (Levene Median test) and normality test (Kolmogorov-Smirnov test) (SigmaStat 3.5., San Jose, CA). After confirmation that the data did not violate the assumptions of parametric testing, a two-tailed Student's t-test was used (GraphPad Prism 5, La Jolla, CA). Variance in all graphs represents standard error of the mean (SEM).

Results

Generation of human APOE haploinsufficient mouse models

To directly examine the effect of decreasing human apoE levels on Aβ and amyloid deposition, we generated mouse models that have either one or two copies of the human *APOE3* or *E4* gene along with the *APPswe/PS1(L166P)* transgene (*APPPS1*-21 mice). *APOE* knock-in mouse models that express human apoE isoforms under the control of endogenous regulatory elements were utilized, eliminating any potentially confounding effects due to the presence of the endogenous murine *Apoe* gene.

First, we assessed the effect of *APOE* gene haploinsufficiency on apoE mRNA and protein levels in the cortex. As expected, apoE mRNA levels decreased by ~50% in the hemizygous *APOE3/−* and *APOE4/−* mice (Fig. 1*A*). Western blot analysis indicated that apoE protein levels were also significantly decreased in the *APOE* hemizygous compared with *APOE* homozygous mice (Fig. 1*B–C*). Consistent with the western blot data, a similar decrease in apoE levels was observed by apoE ELISA for both *APOE3* and *APOE4* isoform mice (Fig.

1D–*E*). There were no significant alterations in the levels of proteins involved in Aβ production (Fig. 1*F*).

Haploinsufficiency of human APOE dramatically inhibits Aβ accumulation

To determine the effect of APOE genotype and dosage on Aβ, we analyzed Aβ accumulation in *APOE* homozygous and hemizygous mice. Significant reductions in insoluble Aβ40 and Aβ42 levels were observed in *APOE3/−* mice, compared with *APOE3/3* mice (Fig. 2*A–B*). *APOE4/−* mice also had a dramatic decrease in insoluble Aβ40 and Aβ42 levels compared to *APOE4/E4* mice (Fig. 2*C–D*). We also analyzed PBS soluble Aβ levels in mice of all genotypes. Soluble Aβ levels were ~ 100-fold less than PBS insoluble Aβ levels. PBS soluble Aβ levels, except Aβ42 levels between *APOE4/4* and *APOE4/−* mice, were not altered by *APOE* haploinsufficiency (*APOE3/3*, Aβ40: 5.82±0.31 pg/mg, Aβ42: 0.77±0.068 pg/mg; *APOE3/−*, Aβ40: 5.27±0.32 pg/mg, Aβ42: 0.77±0.099 pg/mg; *APOE4/4*, Aβ40: 7.49±0.88 pg/mg, Aβ42: 1.39±0.13 pg/mg; *APOE4/−*; Aβ40: 7.90±0.51 pg/mg, Aβ42: 1.12±0.058 pg/mg).

Reduction of Aβ and fibrillar Aβ plaque load in human *APOE* hemizygous mice

To determine the extent of Aβ deposition in *APOE* homozygous and hemizygous mice, brain sections from 3-month-old mice were immunostained with a biotinylated anti-Aβ antibody HJ3.4 (Fig. 3*A–F*). Quantitative analyses of anti-Aβ immunostaining demonstrated that Aβ plaque load was significantly decreased in *APOE3/−* mice compared with *APOE3/3* mice (Fig. 3*C*). The level of Aβ deposition in both *APOE4/4* and APOE4/− mice was also analyzed in the same way (Fig. 3*D–E*). While *APOE4+/+* and *APOE4+/−* mice had significantly greater Aβ deposition than *APOE3+/+* and *APOE3+/−* mice respectively, *APOE4/−* mice had a dramatic reduction in Aβ plaque load, compared with *APOE4/4* mice (Fig. 3*F*). To further characterize the nature of the deposited Aβ plaques, brain sections were stained with X-34 dye that selectively detects only fibrillar, but not diffuse, Aβ deposits. Consistent with the anti-Aβ antibody staining, *APOE3/−* and *APOE4/−* hemizygous mice had significantly less X-34-positive fibrillar plaque load, compared with *APOE3/3* and *APOE4/4* homozygous mice (Fig. 3*G–L*). Taken together, our results clearly demonstrate that haploinsufficiency of *APOE* decreases both total Aβ and fibrillar amyloid deposition.

APOE dosage-dependent modulation of neuroinflammatory response

Aberrant inflammatory responses, such as activation of microglia, are common pathological features in the brains of AD patients. To identify activated microglia in the brain, mouse brain sections were stained with anti-CD45 antibody, a marker of microgliosis. Plaque-associated microglial activation was evident around Aβ deposits in *APPPS1*-21 mice with APOE isoforms (Fig. 4*A–B and C–D*). Quantitative analysis of microgliosis indicated a ~ 95% decrease in the CD45-positive activated microglial load in *APOE3/−* mice, compared with *APOE3/3* littermates (Fig. 4*E*). A similar dramatic phenotype was observed between *APOE4/4* and *APOE4/−* mice (Fig. 4C–*D*). There was a ~97% reduction in microgliosis in *APOE4/−* mice (Fig. 4*F*). These findings indicate that the reduction of Aβ and amyloid formation by APOE haploinsufficiency is closely associated with an attenuation of the microglial neuroinflammatory response. We also analyzed the levels of presynaptic and postsynaptic proteins. Analysis of synaptophysin, glutamate receptor (GluR) 2/3/4, NDMA receptor 2b, and postsynaptic density protein 95 (PSD95) levels indicated there was no significant alteration in these proteins between genotypes or *APOE* dosage levels (Fig. 4*G*).

Discussion

We tested whether decreasing human apoE levels will increase or decrease Aβ accumulation *in vivo*. Given the importance of this question, there have been numerous attempts to

investigate the effects of modulating apoE levels on AD pathogenesis (Ashford, 2002; Rebeck et al., 2002; Teter et al., 2002). However, studies ranging from *in vitro* Aβ aggregation, *ex vivo* Aβ assays, and pharmacological approaches have provided conflicting results (Kim et al., 2009a). Most *in vivo* studies also provided limited information regarding the effect of human apoE modulation, since they indirectly inferred the effect of human apoE based on the manipulation of mouse apoE. To overcome some of the limitations of previous studies, we took a genetic approach by generating human *APOE* homozygous or hemizygous mice without the presence of the confounding endogenous mouse *Apoe* gene. Aβ depositing mouse models with two copies (*APOE3/3* or *APOE4/4*) or one copy (*APOE3/−* or *APOE4/−*) of each *APOE* isoform were successfully generated. *APOE3/−* and *APOE4/−* mice had significantly less Aβ accumulation, amyloid deposition, and microgliosis, compared with the respective homozygous littermates. Thus, decreasing human apoE levels may be an attractive therapeutic strategy for attenuating Aβ deposition and its downstream effects in AD.

Given the critical role of Aβ in AD pathogenesis, the effect of apoE isoforms on Aβ deposition has been extensively investigated. *APOE* isoform-dependent amyloid deposition has been consistently observed in humans and Aβ depositing mouse models (Holtzman et al., 2000; Bales et al., 2009; Reiman et al., 2009; Morris et al., 2010). Consistent with previous studies with other APP mouse models, we also observed *APOE* isoform-dependent effects on Aβ deposition in the *APPPS1*-21 mouse model (E4>E3). Since apoE levels differ between *APOE4/4* and *APOE3/3* with levels of apoE4 being lower than apoE3, it is tempting to speculate that the difference in Aβ accumulation between the apoE isoforms is due to differences in apoE3 versus apoE4 levels. However, the structure of apoE3 and apoE4 differs significantly from each other (Mahley et al., 2006), and the observed difference in Aβ deposition cannot be simply attributed to apoE4 levels being lower than apoE3. For example, *APOE4/E4* mice have greater amyloid deposition than *APOE3/−* mice despite the fact that apoE4 levels are higher in the *APOE4/E4* than in the *APOE3/−* mice. *APOE* gene dosage-dependent amyloid deposition could be due to an effect of an alteration in Aβ clearance and/or aggregation. Several studies demonstrated that apoE isoforms can differentially affect Aβ clearance (Deane et al., 2008; Jiang et al., 2008; Castellano et al., 2011). ApoE isoforms may be slowing Aβ clearance in an apoE-isoform-dependent manner (E4>E3>E2). This idea is supported by the fact that mice lacking apoE have accelerated Aβ clearance from the brain relative to apoE-expressing mice (DeMattos et al., 2004). Another potentially important mechanism is direct facilitation of Aβ fibrillogenesis by an ApoE and Aβ interaction. While there are a few conflicting reports (Kim et al., 2009a), several groups have reported that all three apoE isoforms increase Aβ fibrillogenesis, with the effect being most exacerbated with ApoE4 and the least with ApoE2 (Ma et al., 1994; Wisniewski et al., 1994; Castano et al., 1995).

Given the isoform-dependent effect on AD pathology, one therapeutic approach is to convert apoE4 to an apoE3-like protein by using a small molecule (Mahley et al., 2006). Disrupting the apoE-Aβ interaction could be another attractive therapeutic approach to prevent apoE-mediated Aβ aggregation (Sadowski et al., 2004). Previous studies suggested that modulation of apoE levels and lipidation state, independent of *APOE* genotype, may be another potential strategy to decrease Aβ accumulation (Cao et al., 2006; Wahrle et al., 2008; Kim et al., 2009b). Interestingly, deletion of ATP-binding cassette, sub-family A1 *(Abca1)* and liver x receptor (*Lxr*) genes decreased apoE levels but increased or had no effect on Aβ deposition (Hirsch-Reinshagen et al.,2005; Koldamova et al., 2005; Wahrle et al., 2005; Zelcer et al., 2007). In addition, several studies have shown that activation of *Lxr* increases mouse apoE and decreases Aβ levels (Jiang et al., 2008). However, it is difficult to interpret the effect of altering apoE level in these studies, since modulation of *Abca1* and *Lxr* also influences the lipidation state of apoE. Whether reducing human apoE levels

without affecting its lipidation state will promote or attenuate Aβ accumulation was not clear from previous studies. Our study demonstrates the beneficial effect of decreasing apoE levels on Aβ accumulation. Consistent with our finding, low levels of apoE were strongly associated with low cerebral Aβ load in humans (Darreh-Shori et al., 2010). *APOE* gene promoter studies also suggest that higher levels of apoE, regardless of isoform status, increase the risk of developing AD (Laws et al., 2003). APOE may also influence risk for AD by mechanisms that are not related to Aβ. We found no clear differences in pre- and post-synaptic proteins in *APPPS1*-21 mice of different *APOE* genotypes and doses. This, however, does not rule out the possibility the structural synaptic changes might be present that have been observed by others (Dumanis et al., 2009). Our results in which apoE levels are altered by changing gene dosage suggest that strategies to decrease apoE levels in the brain should be further explored for the prevention/treatment of AD. Changing apoE levels in an adult in the context of 2 copies of *APOE* is different than having a lifelong genetic alteration of *APOE* dosage as in our experiments. It will be important to see in the former context whether altering apoE levels in an adult with 2 endogenous copies of *APOE* has similar or different effects as we report herein.

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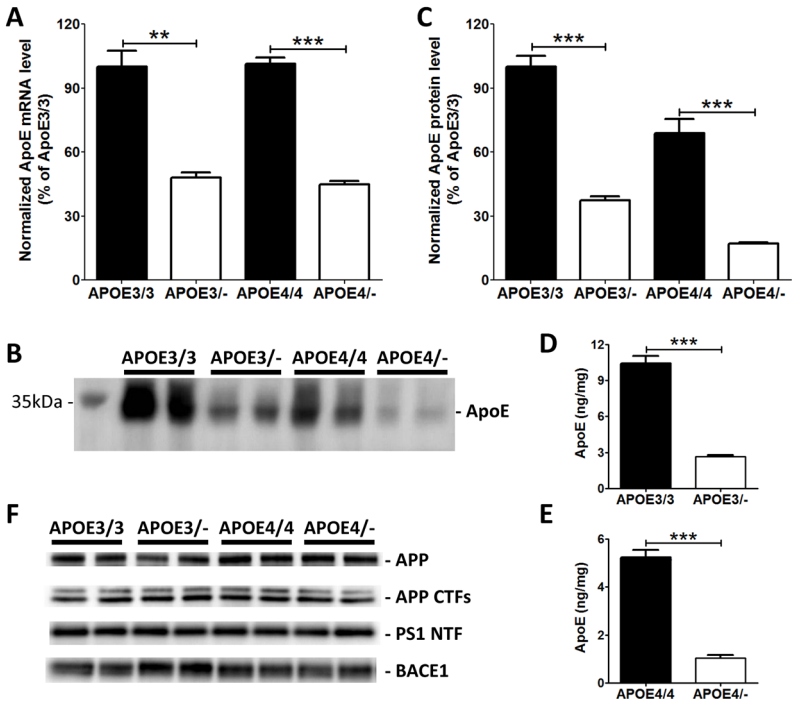

Figure 1. Reduction of apoE levels in human *APOE* haploinsufficient mice

Cortex from *APOE* homozygous (*APOE3/3* and *APOE4/4*) and hemizygous (*APOE3/−* and *APOE4/−*) mice were used to measure apoE mRNA and protein levels. ***A,*** ApoE mRNA levels were measured by quantitative real-time PCR. ***B, C,*** Levels of PBS-soluble apoE were assessed by probing a membrane with anti-apoE antibody (***B***). Quantitative analyses of western blots were performed with tubulin normalization (***C***) (n=4 per genotype). ***C, D,*** To validate the western blot data, apoE levels were also measured by using an apoE-specific ELISA from *APOE3/3* and *APOE3/−* mice (***C***) and *APOE4/4* and *APOE4/−* mice (***D***) (n=9–18 per genotype). ***E,*** Levels of APP, APP CTFs, PS1 NTF, and BACE1 proteins were measured by Western blotting (n=4 per genotype). Levels of these proteins after normalizing with tubulin signals were not significantly altered by *APOE* haploinsufficiency. All graphs represent values in mean ± SEM.

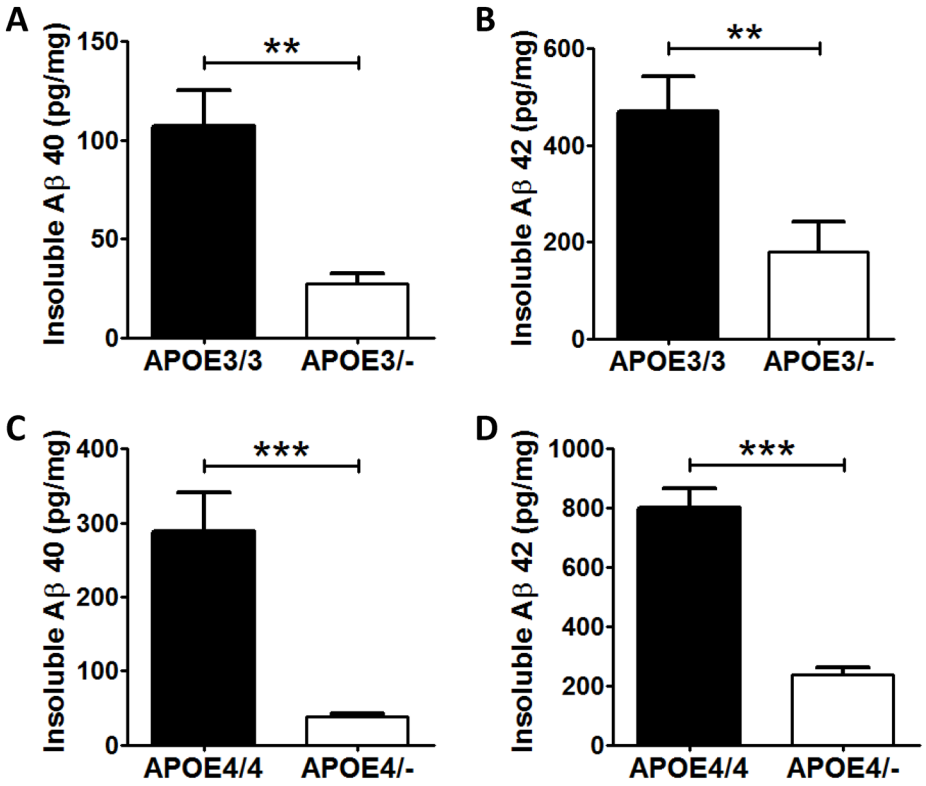

Figure 2. Decreased Aβ accumulation in human *APOE* haploinsufficient mice

Cortical tissues from 3-month-old *APOE* homozygous and hemizygous mice were sequentially homogenized with PBS and RIPA. Aggregated forms of Aβ in the RIPA-insoluble pellet were solublized with 5M guanidine HCl buffer. ***A, B,*** RIPA-insoluble Aβ40 (***A***) and Aβ42 levels (***B***) were measured from *APOE3/3* and *APOE3/−* mice. ***C, D,*** Similarly, RIPA-insoluble Aβ40 (***C***) and Aβ42 levels (***D***) were measured from *APOE4/4* and *APOE4/−* mice. (n=12–20 per genotype)

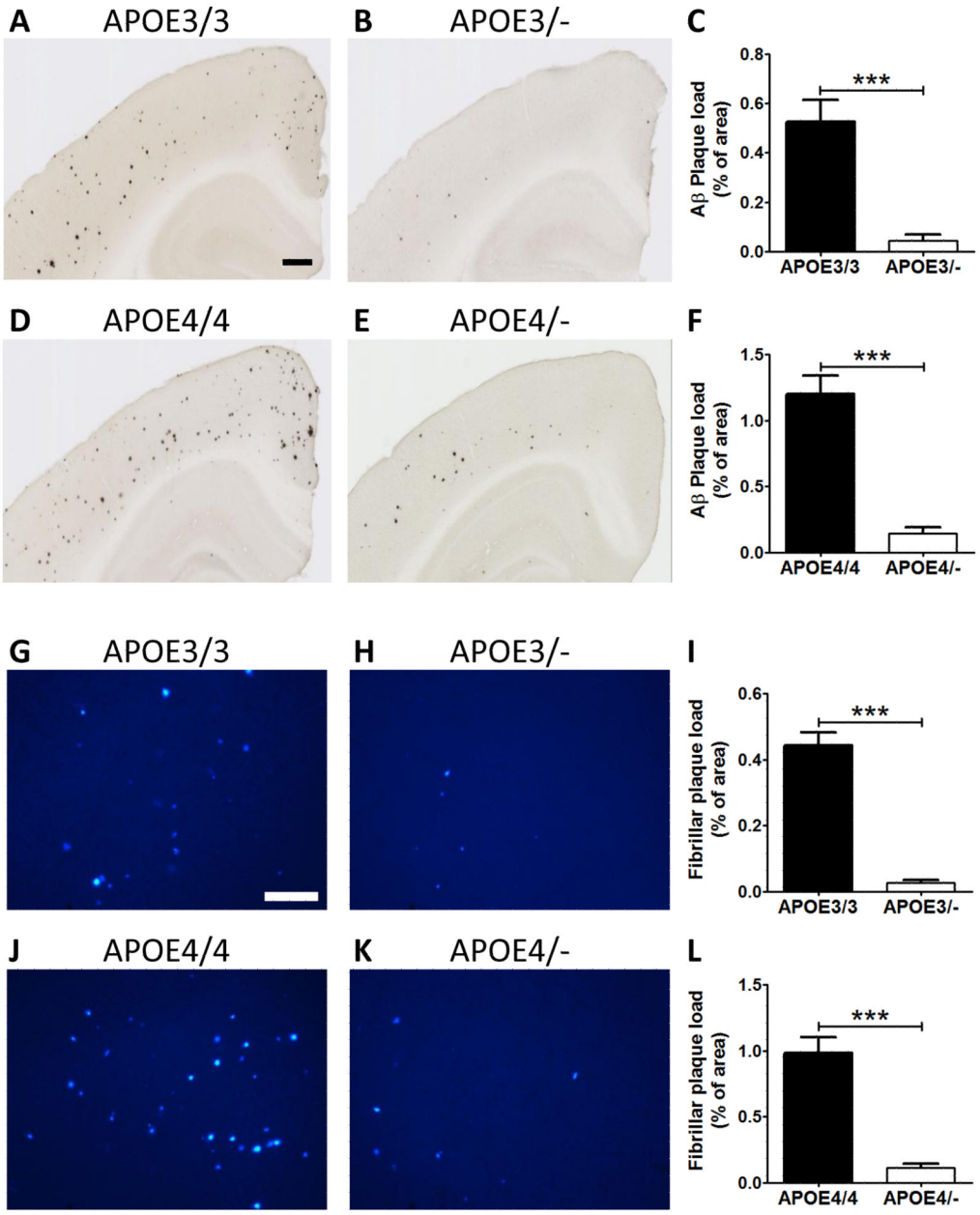

Figure 3. Haploinsufficiency of human *APOE* leads to reduction of Aβ and fibrillar Aβ plaque deposition

A, B, D, E, Brain sections from 3-month-old *APOE* homozygous (***A, D***) and hemizygous (***B, E***) mice were immunostained for amyloid with anti-Aβ antibody (HJ3.4-biotin). Scale bar: 400μm. ***A, B, C,*** The extent of Aβ deposition detected by HJ3.4-biotin antibody was quantified (***C***) from cortex of *APOE3/3* (***A***) and *APOE3/−* mice (***B***). ***D, E, F,*** The extent of Aβ deposition detected was quantified (***F***) from cortex of *APOE4/4* (***D***) and *APOE4/−* mice (***E***). ***G, H, J, K,*** Brain sections from 3-month-old *APOE* homozygous (***G, J***) and hemizygous (***H, K***) mice were stained with X-34 dye that recognizes only fibrillar plaques. Scale bar: 200μm. ***G, H, I,*** Fibrillar plaque load detected by X-34 dye was quantified (***I***) from cortex of *APOE3/3* homozygous (***G***) and APOE3/− mice (***H***). ***J, K, L,*** The extent of

fibrillar plaque load was also analyzed (***L***) from cortex of *APOE4/4* (***J***) and *APOE4/−* mice (***E***). (n=12–20 per genotype)

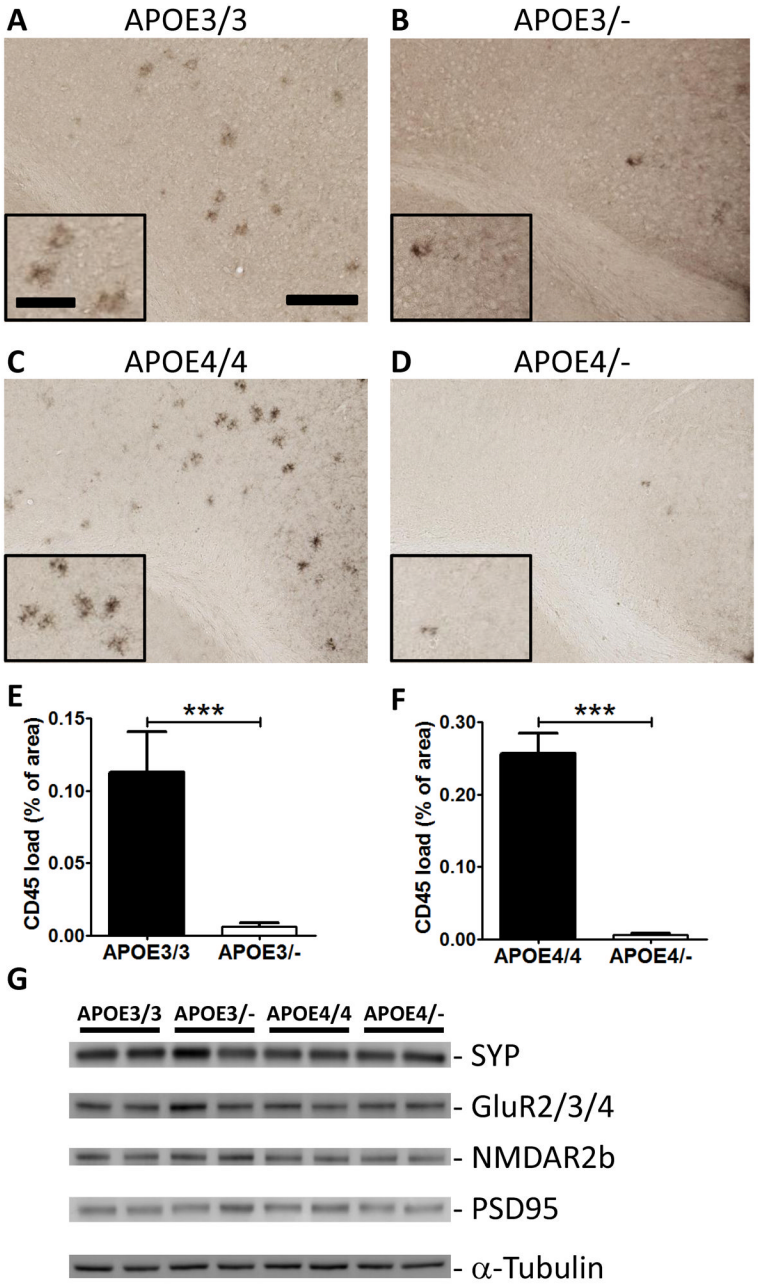

Figure 4. Attenuation of microgliosis in human *APOE* haploinsufficient mice

A, B, D, E, Brain sections from 3-month-old male APOE homozygous (***A, D***) and male hemizygous (***B, E***) mice were immunostained with an antibody against activated microglial CD45. ***A, B, C,*** The percent area covered by CD45 staining was quantified (***C***) from the cortex of *APOE3/3* (***A***) and *APOE3/−* mice (***B***). ***D, E, F,*** The extent of microgliosis was also quantified (***F***) from the cortex of *APOE4/4* (***D***) and *APOE4/−* mice (***E***). Scale bar: 200μm for lower magnification and 90μm for higher magnification. (n=6–12 male mice per genotype). ***G,*** Levels of synaptophysin, glutamate receptor (GluR) 2/3/4, NDMA receptor 2b, and postsynaptic density protein 95 (PSD95) levels were measured by Western blotting (n=4 per genotype). Levels of these proteins after normalizing with tubulin signals were not significantly altered by *APOE* genotype or haploinsufficiency.