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Hyper-expression of human apolipoprotein E4 in astroglia and neurons does not enhance amyloid deposition in transgenic mice

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Recent studies in mice have clearly demonstrated that eliminating Apo E alters the rate, character and distribution of Aβ deposits. In the present study, we asked whether elevating the levels of Apo E can, in a dominant fashion, influence amyloid deposition. We expressed human (Hu) Apo E4 via the mouse prion protein promoter, resulting in high expression in both astrocytes and neurons; only astrocytes efficiently secreted Hu Apo E4 (at least 5-fold more than endogenous). Mice hyper-expressing Hu Apo E4 developed normally and lived normal lifespans. The co-expression of Hu Apo E4 with a mutant amyloid precursor protein (APP) (Mo/Hu APPswe) or mutant APP and mutant presenilin (PS1dE9) did not lead to proportional changes in the age of appearance, relative burden, character or distribution of Aβ deposits. We suggest that these data are best explained by proposing that the mechanisms by which Apo E influences Aβ deposition involves an aspect of its normal function that is not augmented by hyper-expression.

INTRODUCTION

Alzheimer's disease (AD) is characterized by neurofibrillary tangles and parenchymal deposits of β-amyloid peptides (Aβ1–40 and Aβ1–42) (1), the latter of which are generated via endoproteolytic cleavage of an integral membrane protein termed the amyloid precursor protein (APP) (2–4). Much evidence suggests that Aβ deposition is a critical event in the pathogenesis of AD. Mutations in APP, which occur in cases of familial early-onset AD (FAD), all influence APP processing to increase the production of Aβ1–42 peptides (5–8). Presenilin 1 with mutations linked to FAD catalyzes enhanced production of Aβ1–42 peptides (9–13). The brains of transgenic mice that co-express FAD mutant HuPS1 and Hu APP have an earlier age of appearance of Aβ deposits than mice expressing APP alone or doubly transgenic mice co-expressing wild-type HuPS1 and APP (10,14,15). Immunocytochemical studies of brains from AD and Down's syndrome patients (16–19), and mass spectrometric analysis of purified amyloid deposits (20), have demonstrated that Aβ1–42 is the major peptide deposited. Collectively, these studies demonstrate that Aβ deposition is capable of initiating the pathogenesis of AD.

The presence of an Apo E4 allele increases the risk of developing AD (21,22). In both AD and Down's syndrome, Apo E4 alleles are associated with an earlier age of dementia and an increased amyloid burden (23–30). Additional evidence for a modulatory role of Apo E in Aβ deposition comes from studies of transgenic mice. Ablation of Apo E in transgenic mice, expressing APP_{V717F}, prevented the appearance of compact, thioflavin-S+ plaques, and shifted the localization of diffuse plaques within the hippocampus when compared to APP_{V717F} mice expressing endogenous Apo E (31–33). Thus, there is growing evidence that Apo E can modulate the deposition of Aβ.

Our approach to examining the role of Apo E in Aβ deposition has been to express human (Hu) Apo E4 at very high levels in a setting where the expression of endogenous mouse Apo E is undisturbed. Our strategy to achieve high levels of expression employed a vector derived from the mouse prion protein gene; previous experience with the vector led us to believe that its use would have a high probability of generating mice that hyper-express Hu Apo E4 in brain. We successfully generated transgenic mice that express high levels of Hu Apo E4. Although the transgene was expressed in both astrocytes and neurons, only astrocytes efficiently secreted Apo E4. We estimate that CNS astrocytes secrete Hu Apo E4 at more than

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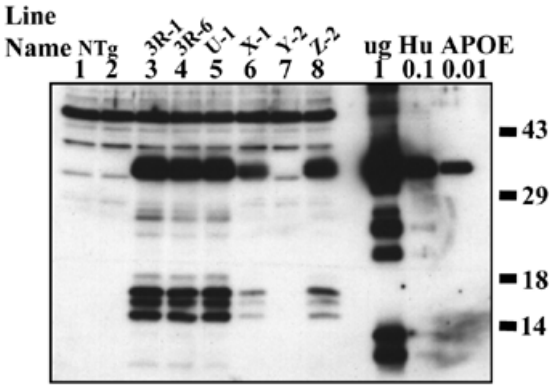

Figure 1. Apo E4 is expressed at high levels in the brains of transgenic mice. Hemibrains from Apo E4 mice were homogenized in PBS using a hand-held polytron. Crude homogenates were then extracted in SDS–PAGE buffer and electrophoresed for immunoblot with antisera that are specific for Hu Apo E (Calbiochem). Each lane (1–8) contains 100 μg of total protein. Purified Hu Apo E (Calbiochem) was co-electrophoresed for comparison (1, 0.1, 0.01 μg). Lines 3R-1 and U-1 express Apo E4 at levels that approach 100 ng per 100 μg of total brain protein.

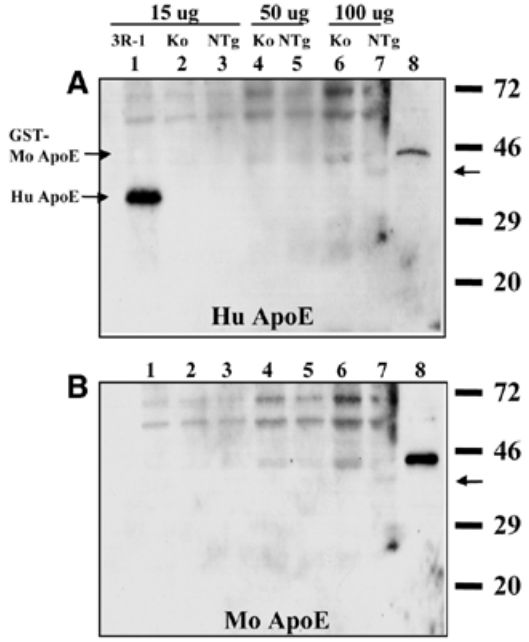

Figure 2. Hyper-expression of Hu Apo E4 in transgenic and non-transgenic animals. (**A** and **B**) Hemibrains from Apo E4 transgenic (lane 1), and Apo E knockout mice (lanes 2, 4 and 6), and non-transgenic (lanes 3, 5 and 7) were homogenized in PBS, then centrifuged at 200 000 *g* for 30 min. Fifteen (lanes 1, 2 and 3), 50 (lanes 4 and 5) and 100 μg (lanes 6 and 7) of protein were electrophoresed in the absence of mercaptoethanol (mouse IgG remains cross-linked and migrates at 150 kDa). Lane 8 contains 25 ng of GST–Mo Apo E fusion protein (see Materials and Methods). The blots (A) were probed with a mouse monoclonal antibody that favors Hu Apo E (Boehringer Mannheim). No Apo E could be detected in soluble fractions prepared from non-transgenic mice with either the monoclonal antibody to Hu Apo E or with a monoclonal antibody specific to mouse Apo E (Biodesign International, ME). Hence, in the high-speed supernatant fractions of mouse brain, which should be enriched for secreted Apo E, the level of mouse Apo E is <25 ng per 100 μg of total protein in the soluble fraction of brain homogenate.

five times the normal levels of murine Apo E. These mice were mated to mice expressing Mo/Hu APPswe and to mice with accelerated Aβ deposition, which co-express Mo/Hu APPswe and Hu PS1dE9 (exon 9-deleted variant). In mice expressing both Apo E4 and Mo/Hu APPswe, the levels of Aβ1–42 were slightly elevated, but the age of appearance of Aβ deposits was not significantly accelerated. Moreover, the age of appearance, relative burden, character (diffuse versus neuritic) and distribution of Aβ deposits in Mo/Hu APPswe/PS1dE9 mice was unaltered by the presence of high levels of Hu Apo E4. We also note that mice expressing Hu Apo E4 in the presence of high levels of Aβ deposition did not develop alterations in tau phosphorylation or tau aggregates. Using levels of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) as surrogate markers of neurodegeneration, we note that the presence of Hu Apo E4 does not appear to heighten the toxicity of Aβ in the murine CNS. Thus, overall, the hyper-expression of Hu Apo E4, in the presence of endogenous mouse Apo E, does not lead to obvious changes in AD-related neuropathology in transgenic mouse models of β-amyloid deposition. These data suggest that the mechanism by which Apo E4 influences Aβ deposition does not involve a direct interaction between Apo E4 and Aβ peptides. Instead, we suggest that these data are best explained by proposing that the mechanisms by which Apo E influences Aβ deposition involve an aspect of its normal function that is not augmented by hyper-expression.

RESULTS

Apo E4 transgenic mice

Immunoblot analysis was used to identify lines of transgenic mice that express high levels of Hu Apo E4. Using purified Hu Apo E4 as a standard, we estimate that two of the lines of mice we produced (U1 and 3R1) produce Hu Apo E4 at levels that approach 100 ng per 100 μg of total protein (Fig. 1, lanes 3 and 5). Notably, the hyper-expression of Apo E4 is associated with the appearance of a trio of 15–17 kDa Apo E-associated polypeptides that appear to be proteolytic fragments of Apo E (Fig. 1, lanes 3–5, 6 and 8). It is unclear whether these fragments are related to a 22 kDa, apparently toxic, Apo E fragment described by others (34). The immunoblots shown in Figure 1 also demonstrate a number of high molecular weight bands, visible at the top of the immunoblot (Fig. 1, lanes 1–8), that appear to be cross-reacting proteins as they are present in both non-transgenic (lanes 1 and 2) and Apo E4 transgenic mice (lanes 3–8).

To determine the level of Hu Apo E4 relative to endogenous Apo E, we collected the supernatants from 200 000 *g* centrifugations of homogenates from Apo E4 transgenic animals, non-transgenic littermates and Apo E knockout mice (Fig. 2). These fractions should include the portion of Apo E polypeptides that have been secreted into the extracellular space. An antiserum raised against Hu Apo E demonstrated high levels of Apo E4 in the supernatant prepared from the brains of the transgenic mice (Fig. 2A, lane 1). Although this antiserum cross-reacts with a fusion protein of glutathione synthetase (GST) and mouse Apo E (GST–Mo Apo E) (Fig. 2A, lane 8), we could not detect an Apo E-specific signal in similarly

prepared supernatants from non-transgenic mice (Fig. 2A, lanes 3, 5 and 7). Supernatant fractions from Apo E knockout mice were prepared in parallel to provide a control for non-specific immunoreactivity (lanes 2, 4 and 6). Lane 8 of Figure 2B contains 25 ng of purified GST–Mo Apo E as a standard to calculate the level of mouse Apo E. Thus, 100 µg of the soluble fraction of brain homogenates from non-transgenic mice contains <25 ng of endogenous Apo E. In contrast, we demonstrate in Figure 1, that 100 µg of total brain homogenate from our transgenics contains ~100 ng of Hu Apo E4.

Previous studies of the expression of transgenes under the influence of the prion promoter have demonstrated high levels of expression in the nervous system and in muscle, with lesser levels of expression in most other tissues (35–37). In brain, *in situ* hybridization studies have demonstrated high levels of expression in neurons (35). Astroglia are known to express prion protein (38,39) and since astroglia are the normal source of Apo E in the brains of mice (40,41), we examined primary cultures of astrocytes and neurons to determine whether astroglia express the Apo E4 transgene and whether the protein is secreted. Conditioned medium from cultures of both neurons (Fig. 3A, lanes 6 and 7) and astrocytes (Fig. 3A, lanes 3 and 4) contained Hu Apo E4. However, clearly, the level of secretion from astrocytes greatly exceeded the level from neurons. Moreover, the level of secreted Hu Apo E4 was significantly higher than that of endogenous Apo E since no signal was detected in medium from cultures of non-transgenic animals (Fig. 3A, compare lanes 3 and 4 to 5). Equal amounts of Hu Apo E4 and a GST–Mo Apo E fusion protein demonstrated that the antibody used is capable of recognizing both human and mouse Apo E. Analyses of cell lysates from these cultures demonstrated Hu Apo E4 was synthesized by both neurons and astroglia (Fig. 3B); however, the expression in neurons was at least 5-fold greater than that of astrocytes (with equal loading of protein). Immunoblots for glial fibrillary acid protein (GFAP) confirmed the presence of astrocytes in the glial cultures (Fig. 3C), but also demonstrated a slight contamination of the neuronal cultures with glia; this is often unavoidable when neuronal cultures are produced from neonatal animals as was the case in these experiments. Hence, it is possible that all of the Apo E4 detected in medium from neuronal cultures may be derived from contaminating glia cells. Nevertheless, it is quite clear that neurons do not efficiently secrete Apo E4. Collectively, the data indicate that in our transgenic animals, the majority of Hu Apo E4 that would be secreted into the extracellular space is derived from astroglia.

Immunoblot analyses of Apo E4 levels in our transgenic animals were assessed at several ages and at several different generations. In no case did we observe a decrease in the levels of Apo E4 expression (data not shown). Throughout the lifespan of the animals, the levels of expression remained high and there was no indication of silencing of the transgene in successive generations (data not shown).

Neuropathological evaluations of Apo E4 transgenic mice failed to reveal obvious neuropathological lesions. There were no obvious reductions in the numbers of neurons in the cortex and hippocampus (data not shown). Immunostains with antisera to GFAP failed to demonstrate an astrocytic response (data not shown) and neurofibrillary tangles were not demonstrated by silver impregnation. Over the course of 2 years, there was no evidence of increased mortality and transgenic animals remained indistinguishable from non-transgenic littermates.

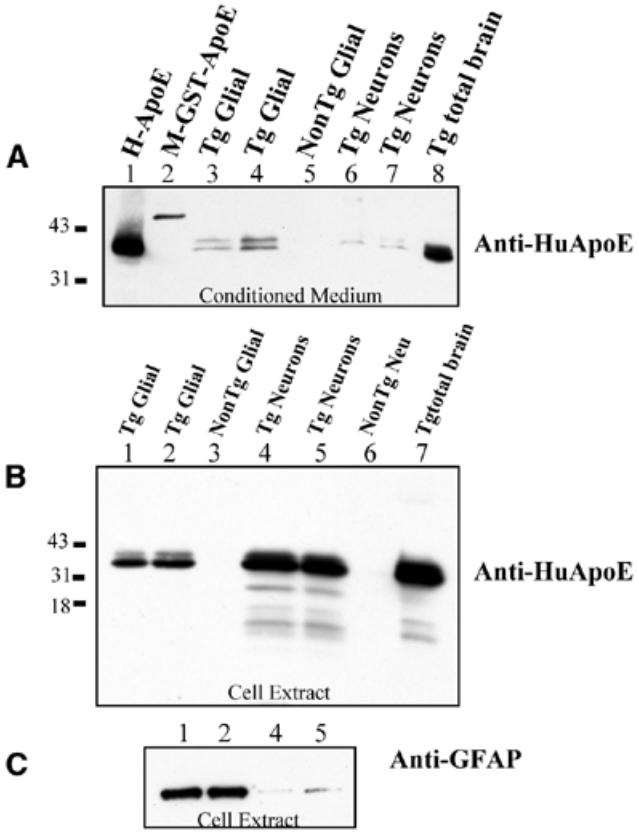

Figure 3. Expression of Hu Apo E4 in primary cultures of astrocytes and neurons from transgenic animals. Primary cultures enriched in either neurons or astrocytes were prepared as described in Materials and Methods. (**A**) Medium from 2 day incubations, without changing, were collected from cultures generated from transgenic (Apo E4 only) or non-transgenic animals. A 10–20 µl aliquot of culture medium was mixed with 2× sample buffer, boiled, and electrophoresed in 10% polyacrylamide gels. Loading of medium was normalized by the protein content of detergent extracts of cell pellets. Blots were probed with the anti-Hu Apo E antibody described in Figure 2. Fifty nanograms of purified Hu Apo E and GST–Mo Apo E were co-electrophoresed for comparison. (**B**) Detergent extracts of total cell pellets were prepared as described in Materials and Methods. Five micrograms of cellular protein was electrophoresed in 10% gels and transferred to a nitrocellulose membrane for immunoblotting with a monoclonal antibody to Hu Apo E (Fig. 2). (**C**) The relative purity of glia and neuronal cultures were assessed by immunoblotting to GFAP (Dakko, Carpintera, CA). The numbers above the panel correspond to the sample numbers above (B). A slight contamination of the neuronal cultures with astroglia is noted.

Influence of Apo E4 on Aβ deposition

To examine whether Apo E4 expressed at the levels present in our mice would influence Aβ deposition, we mated our Apo E4 transgenic mice with mice expressing Mo/Hu APPswe. Our initial study of offspring from these crosses focused on young animals so that we could discern whether the presence of Apo E4 impacts on the production/metabolism of Aβ (independent of whatever impact it may have on deposition). Measurements of the levels of total Aβ1–40 and Aβ1–42 in the brains of young transgenic mice revealed no significant change in the

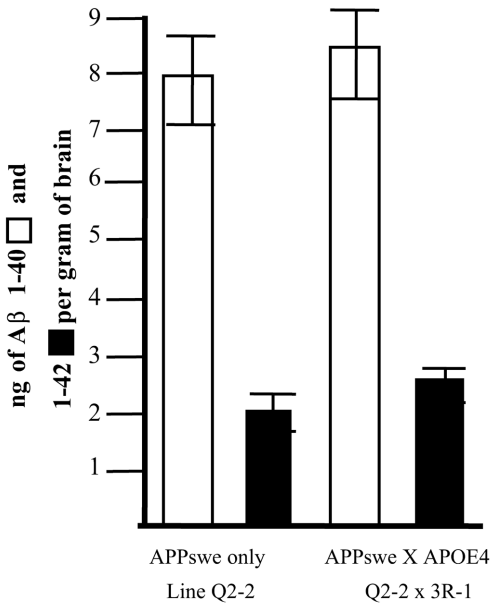

Figure 4. Levels of Aβ 40 and 42 in APPswe and APPswe/Apo E mice. The levels of Aβ peptides in the brains of mice co-expressing Apo E4 and APPswe were measured using an immunoprecipitation and immunoblot strategy. In this study we examined the levels of Aβ 40 and 42 in young animals derived from crosses APPswe (line Q2-2) × Apo E4 (line 3R-1). Line Q2-2 is homozygous for the APPswe transgene which facilitates the production of double transgenic animals. However, the levels of APPswe expression in these animals are slightly lower than that of line C3-3 and they do not develop Aβ deposits (unpublished observation). Animals were harvested at 2–4 months of age. A small increase, which just reached statistical significance ($P < 0.05$), in Aβ 42 levels was observed in the APPswe/Apo E4 mice.

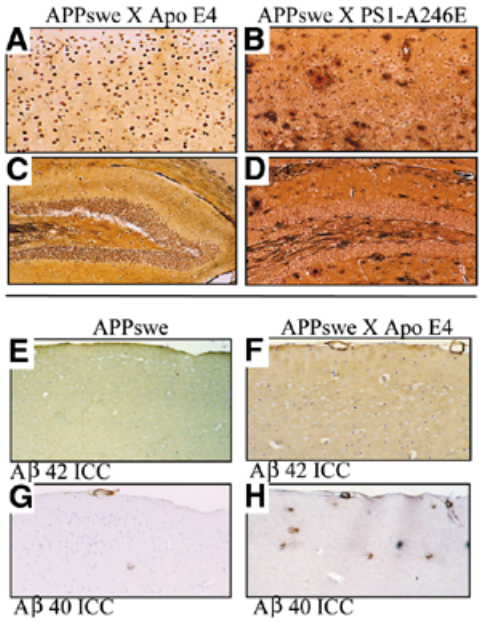

Figure 5. Hyper-expression of Hu Apo E4 does not accelerate the age of appearance of Aβ1–42 deposits in mice co-expressing Mo/Hu APPswe. Eighteen-month-old mice expressing APPswe and Apo E4 were perfused as described in Materials and Methods. Sections (10 μm) were then stained by silver impregnation as described in Materials and Methods (**A–D**). As compared to animals that co-express APPswe with mutant PS1-A246E, where the amyloid burden is relatively high (B and D), mice that co-express APPswe with Apo E4 show neither diffuse nor compact Aβ deposits. Similarly, immunostaining with antibodies specific for Aβ1–42 failed to detect either diffuse or compact Aβ deposits (**E** and **F**). In a subset of APPswe/Apo E4 mice, the number of vessels showing Aβ1–40 immunoreactivity increased (**G** and **H**).

levels of Aβ1–40 in the brains of the Apo E4 × Mo/Hu APPswe mice as compared to mice expressing Mo/Hu APPswe alone (Fig. 4). A slight increase in Aβ1–42 was noted in APPswe/Apo E4 mice as compared to APPswe mice (Fig. 4). However, a statistical evaluation of these values revealed only marginal significance ($P < 0.05$). The low magnitude of the increase (<10%) and the low significance lead us to conclude that the presence of Hu Apo E4 has virtually no impact on the production/metabolism of Aβ1–40 or Aβ1–42.

Neuropathological analysis of Apo E × APPswe mice aged 18–20 months failed to demonstrate significant numbers of neuritic, or diffuse, Aβ deposits (Fig. 5). Sections stained by silver impregnation (Fig. 5A–D) and by immunocytochemistry with antibodies specific for Aβ1–42 peptides (Fig. 5E and F) failed to demonstrate a significant increase in the burden of compact or diffuse deposits, as compared to mice expressing APPswe, alone. In a subset of APPswe/Apo E4 mice, an increased frequency of Aβ1–40 immunoreactive vessels was noted (Fig. 5H). However, this phenotype was not fully penetrant as only 40% of the double transgenic animals demonstrated Aβ1–40 immunoreactive vessels.

To investigate further whether Apo E4 can influence Aβ deposition, we mated the Apo E4 mice to mice that co-express Mo/HuAPPswe and the exon-9-deleted variant of Hu presenilin 1 (PS1dE9). Both APPswe/PS1dE9 and APPswe/PS1dE9/Apo E4 mice begin to show argentophilic Aβ deposits by 4–6 months of age (compare Fig. 6A and B to C and D). Previous studies of Apo E knockout mice have demonstrated that the distribution of Aβ deposition within the hippocampus is altered in the absence of Apo E (33). In the hippocampus of both APPswe/PS1dE9 and APPswe/PS1dE9/Apo E4 mice, deposits of Aβ were most abundant in the molecular layers of the dentate gyrus (Fig. 6). To quantify whether the presence of Apo E4 impacts on the character of Aβ deposits (neuritic versus diffuse), we stained hippocampal sections with antibodies to PS1 (marks neurites), and counted the numbers of immunoreactive structures in representative sections from three triple transgenics versus four double transgenics (Table 1). The numbers of argenotphilic deposits in hippocampus were similar in both genotypes, as was the relative abundance of compact versus diffuse deposits. Using PS1 immunocytochemistry to mark neuritic deposits, we found a slight, but not statistically significant, increase in the numbers of neuritic deposits in the triple transgenic mice (Table 1).

To quantify further the impact of Apo E4 on Aβ deposition in these animals, we utilized a recently developed assay for Aβ aggregates that involves the filtration of tissue homogenates through cellulose acetate membranes, followed by immunodetection (X.Guilian, V.Gonzales and D.R.Borchelt, submitted for publication). Filtration through cellulose acetate has been used as a means to detect aggregates of polyglutamine proteins (42–45). In homogenates of AD brain, aggregates of Aβ are retained on these low-protein-binding filters (Fig. 6E).

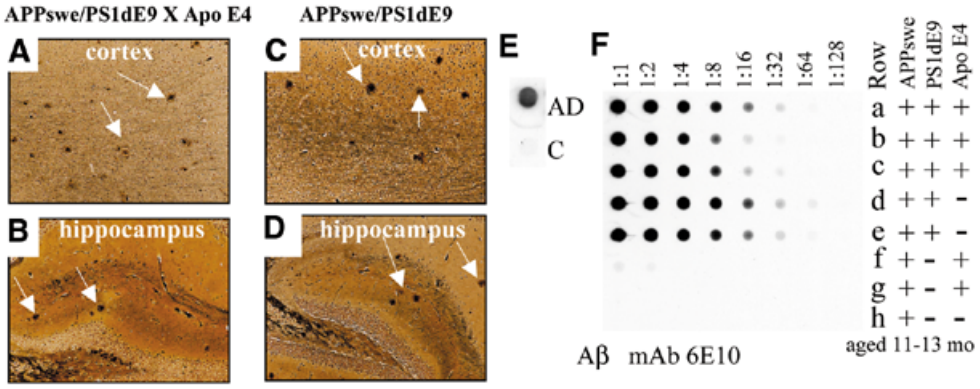

Figure 6. The presence of Hu Apo E4 does not markedly increase the burden of Aβ deposits in APPswe/PS1dE9 mice. (**A**–**D**) The burden of Aβ deposition in the brains of 6-month-old APPswe/PS1dE9 and APPswe/PS1dE9 × Apo E4 mice was examined by silver stain (see Materials and Methods). Representative examples from the cortex and hippocampus are shown. In total, the brains of five triple transgenic and five double transgenic brains were examined. Although there is some variation from one animal to the next, there was no obvious increase in amyloid burden in the triple transgenic animals as compared to age-matched littermates that express only APPswe/PS1dE9. (**E**) Brain tissue from an AD case and an age-matched control [C] was extracted in PBS containing 1% SDS and filtered through cellulose acetate as described in Materials and Methods. Aggregates of Aβ trapped by the filter were revealed by immunostaining. (**F**) Cerebral cortexes from transgenic mice (aged 11–13 months) were extracted in PBS with 1% SDS and filtered as described in Materials and Methods. Aggregates of Aβ were revealed by immunostaining with mAb 6E10. The genotypes of each animal are noted to the right of the panel.

Table 1. The co-expression of Apo E4 with APPswe/PS1dE9 does not increase the frequency of neuritic plaques

Genotype	Silver positive (% compact)	PS1 positive neuritic plaques
APPPswe/PS1dE9/Apo E4	16 ± 3 (80%)	36 ± 9
APPswe/PS1dE9	19 ± 10 (80%)	27 ± 16
t-Test	P <0.2	P < 0.12

Sagittal sections through the hippocampus, at ~2 mm from the midline, from three triple transgenic and four double transgenic animals were stained by silver impregnation and by PS1-loop immunocytochemistry (Fig. 9). Counts were made on at least four sections from each animal and averaged.

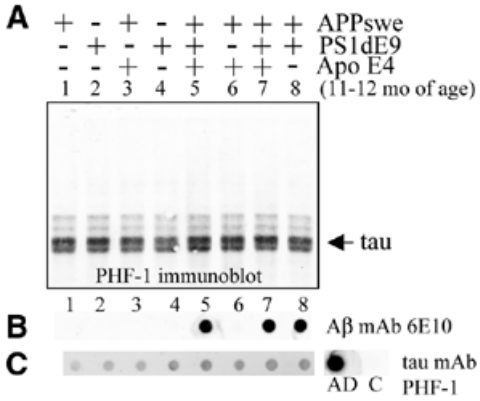

Figure 7. The co-existence of Aβ deposits and Hu Apo E in transgenic mouse brain does not lead to altered phosphorylation or aggregation of tau. (**A**) Cortical regions of transgenic mice (aged 11–12 months) were dissected and homogenized as described in Materials and Methods. Ten micrograms of extracted protein was electrophoresed in 10% SDS–polyacrylamide gels, transferred to nitrocellulose and immunoblotted with the monoclonal antibody PHF-1 (1:200). (**B** and **C**) Homogenates of cortical brain from mice were prepared as described in Figure 6F. 200 µg of total protein from each brain sample was filtered through cellulose acetate. Trapped aggregates of Aβ were then immunostained with the monoclonal antibody 6E10 (1:5000) (B), whereas trapped aggregates of tau were immunostained with the monoclonal antibody PHF-1 (1:200) (C). Examples of tau aggregates trapped from human AD brain as compared to control [C] are shown to the right.

Coupling serial dilution of mouse brain homogenates with cellulose acetate filtration and immunodetection provides a means to accurately quantify and compare the levels of Aβ deposition amongst animals (X.Guilian, V.Gonzales and D.R.Borchelt, submitted for publication). Using this procedure, we demonstrated that the Aβ aggregate loads in 11–13-month-old mice that co-express APPswe/PS1dE9/Apo E4 was similar to that of age-matched mice expressing APPswe/PS1dE9 (Fig. 6F, compare a–c to d and e). Aggregates of Aβ were essentially absent in similarly aged mice expressing APPswe/Apo E4 or APPswe alone (Fig. 6F, f–h). Collectively, these data indicate that Hu Apo E4, expressed under the influence of the prion protein promoter, has little effect on the age of appearance, relative load of Aβ aggregates, character (diffuse versus neuritic) or distribution of Aβ1–40 or Aβ1–42 deposits in mice co-expressing Mo/Hu APPswe and PS1dE9.

Previous studies by others have demonstrated that in the FVB/N strain of mice, the expression of Hu Apo E4, via a neuron specific enolase promoter, leads to an increase in the levels of phosphorylated tau in mice aged >18 months (46). To determine whether the presence of Hu Apo E4, in the context of relatively high amyloid burden, leads to changes in tau metabolism, we extracted total tau from the brains of mice co-expressing APPswe, PS1dE9 and Apo E4 for immunoblot with PHF-1 antibodies, which are specific for phosphorylation of serines 396/404 in tau. Mice expressing all three proteins (Fig. 7, lanes 5 and 7) showed no greater level of tau phosphorylation

at these residues than controls expressing other combinations of transgenes or APPswe and PS1dE9 alone (Fig. 7, lanes 1–4, 6 and 8). Although it is possible that in older mice, the level of phosphorylated tau may increase in mice expressing Hu Apo E4, we find no evidence of a synergistic interaction between deposited Aβ and Apo E4 in altering tau phosphorylation.

To analyze further whether the expression of Apo E4 leads to an increase in tau aggregation, we utilized the cellulose filtration assay described above. Aggregates of Aβ in the brains of transgenic mice are readily retained on cellulose acetate filters (Fig. 7B, genotypes given in A). Similarly, aggregates of tau are retained on cellulose acetate after filtration of human AD brain homogenates and these aggregates can be detected with the PHF-1 monoclonal antibody (Fig. 7C, far right). A similar filtration of homogenates from the transgenic mice yielded no evidence of enhanced aggregation of tau (Fig. 7C, genotypes given in A). Thus, we find no evidence that the presence of Hu Apo E4 leads to significant alterations in the levels of phosphorylated tau or the aggregation of this protein.

In the absence of definitive evidence for changes in pathology, we have not pursued a study of cognitive behavior. The Apo E4 mice were produced in hybrid strains of C57BL/6J and C3H/HeJ and in order to conduct meaningful behavioral studies we would have to backcross these animals to C57BL/6J mice for at least 10 generations (47). Thus, we turned to surrogate markers of cognition to determine whether the presence of Apo E4 augments the toxicity of β-amyloid. The levels of AChE and ChAT were measured in tissue homogenates from ~11–13-month-old mice (Fig. 8). No significant differences in the levels of these enzymes were evident amongst the various genotypes. We conclude that the presence of Hu Apo E4 in a setting of relatively high amyloid burden does not lead to enhanced degeneration of cholinergic neurons, which are the most vulnerable neurons in AD (48–50).

To examine the cellular distribution of Hu Apo E4 in mice with Aβ deposits, sections were immunostained with the monoclonal antibody that reacts preferentially with Hu Apo E (Fig. 2A). In Apo E4 × APPswe/PS1dE9 mice, Apo E4 immunoreactivity was co-distributed with PS1 immunoreactivity (compare Fig. 9A with C and D) in neurites adjacent to the deposits. In contrast, in mice co-expressing Mo/Hu APPswe and PS1dE9 in the presence of endogenous mouse Apo E (Fig. 9B), the neuritic distribution was absent and instead, many deposits showed intense immunoreactivity in the central core of the deposit. The neuritic staining seen in the triple transgenics confirms the expression of Apo E4 in neurons in close proximity to Aβ deposits. Interestingly, in sections from Mo/Hu APPswe/PS1dE9/Apo E4 mice (Fig. 9E), Apo E immunoreactivity appeared as a ring circling the dense core of deposits. In contrast, in mice expressing APPswe and PS1dE9 alone, Apo E immunoreactivity was always concentrated in the dense core of the deposit (Fig. 9B). Whether this new pattern in the triple transgenics reflects a redistribution of endogenous Apo E or the distribution of only Hu Apo E4 is uncertain as the antibody to Hu Apo E4 does cross-react to mouse Apo E (Fig. 2). However, the much higher levels of human protein and the preference of the antibody for Hu Apo E would combine to favor reaction with the human protein in the triple transgenic animals.

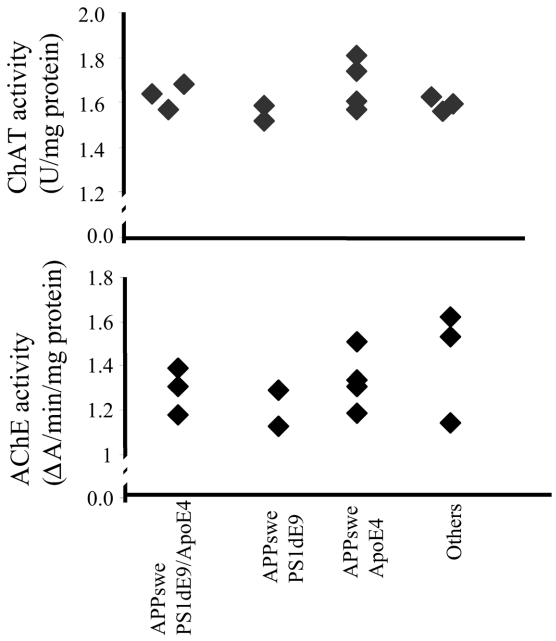

Figure 8. β-Amyloid induced degeneration of cholinergic neurons is not augmented in the presence of Apo E4. The levels of ChAT and AChE were measured as described in Materials and Methods. All of the animals were aged 11–13 months. ChAT activity is given as units per milligram protein (95). AChE activity was measured as the change in absorption at 412 nm per minute per milligram of protein (96).

DISCUSSION

This report describes transgenic mice that express high levels of Hu Apo E4 in both astroglia and neurons. Neurons were found to express higher levels of Hu Apo E4, but these cells do not appear to efficiently secrete the protein. A study of primary astrocyte cultures demonstrated high levels of expression and efficient secretion. We estimate that the levels of Hu Apo E4 that are secreted by astroglia in the brains of our mice are at least five times the normal level of mouse Apo E. These high levels of expression were not associated with obvious neuropathological abnormalities. The levels of Aβ1–40 and Aβ1–42 in the brains of young APPswe/Apo E4 mice were not dramatically elevated or diminished as compared to mice expressing APPswe alone, leading us to conclude that the production and/or metabolism of Aβ peptides was not modified directly by Apo E4. Moreover, the presence of high levels of Apo E4 had no obvious impact on the age of appearance, relative burden, character (diffuse versus neuritic) or distribution of parenchymal Aβ deposits. The co-existence of Aβ deposits and Hu Apo E4 did not lead to increased phosphorylation of tau or to a reduction in the solubility of this protein. In a survey of a limited set of animals, the presence of Apo E4 did not enhance the degeneration of cholinergic neurons.

Apo E and Aβ deposition

Our present study (Fig. 9) and those of others (51,52) support the view that *in vivo* Apo E can associate with Aβ deposits. We demonstrate Hu Apo E4 immunoreactivity around the dense cores of neuritic Aβ deposits (Fig. 9). The antibodies used in

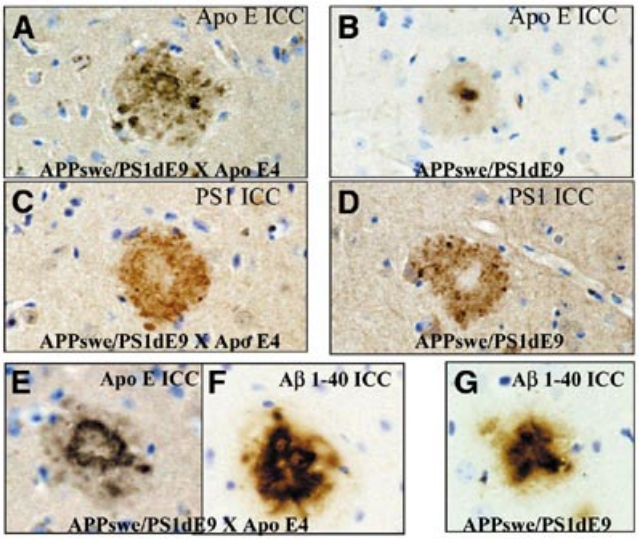

Figure 9. Apo E4 is associated with Aβ deposits in APPswe/PS1dE9 mice. Sections from 6-month-old APPswe/PS1dE9 × APO E4 and APPswe/PS1dE9 mice were immunostained with antibodies to Hu Apo E (**A**, **B** and **E**), Hu PS1 C-terminal fragments (**C** and **D**) and Aβ1–40 (**F** and **G**). In APPswe/PS1dE9 × Apo E4 mice, abundant Apo E immunoreactivity is seen in neurites adjacent to Aβ deposits (A) and in some instances as a ring surrounding the dense core of Aβ deposits (E). A similar ring structure is labeled with antibodies to Aβ1–40 (F and G).

this study show a marked preference for Hu Apo E and levels of human protein are in excess of mouse Apo E. Hence, it seems likely that the immunostaining patterns for Apo E4 that are seen in the triple transgenic animals represent reactivity with the human protein. However, it remains possible that the pattern of immunoreactivity represents a redistribution of the endogenous mouse protein.

Studies in patients suggest that individuals that are homozygous for the E2 allele show the least amyloid deposition, and E4 the most (23,24,30,53–55). CSF levels of Aβ1–42 are elevated in individuals carrying the E4 allele of Apo E (56). Several *in vitro* studies have documented that, as compared to Apo E2, Aβ aggregation and fibrillogenesis is increased in the presence of Apo E4 (57,58), although this observation is disputed (59). The best *in vivo* evidence for Apo E acting as a modulator of Aβ deposition comes from studies in transgenic mice, where it has been demonstrated that the absence of Apo E strongly modifies both the character and location of Aβ deposits (31–33,60). Mice expressing mutant APP in the absence of Apo E do not develop typical compact deposits and, moreover, the remaining diffuse deposits are aberrantly distributed to the cellular layer of the dentate (31,32,60). All of these changes in Aβ deposition can be reversed by expressing Hu Apo E3 and E4, under the influence of the promoter elements of the GFAP gene (33,60) at levels that are roughly equivalent to the normal levels of Apo E in mouse brain. In these later experiments, the appearance of Aβ1–42 deposits was several weeks earlier in mice expressing Hu Apo E4 as compared to Hu Apo E3 (endogenous mouse Apo E is absent). However, in both cases, the onset was later than that which occurs in mice expressing the mutant Hu APP transgene in the presence of only endogenous mouse Apo E. These experiments were interpreted to suggest that Apo E plays an important role in the deposition of Aβ1–42, with E4 possessing a greater potential to promote deposition.

However, our study finds no evidence that hyper-expression of Hu Apo E4 leads to alterations in the rate, burden or distribution of Aβ deposits in transgenic mice. We envision two potential explanations for our findings. One explanation would hold that endogenous mouse Apo E is a far better promoter of Aβ deposition than Hu Apo E4, and thus, the added expression of Hu Apo E4 is of little consequence. However, data from the studies of Holtzman *et al.* (33,60) are not consistent with this explanation as the replacement of murine Apo E with equivalent levels of Hu Apo E4 only slightly delayed the onset of Aβ deposition (2–3 months). A second explanation would hold that the mechanisms by which Apo E influences Aβ deposition involves an aspect of its normal function that cannot be augmented by hyper-expression. In this setting, the mechanism of Apo E action on Aβ deposition cannot involve a direct activity of Apo E protein on the aggregation state of Aβ.

The primary function of Apo E in brain is to modulate the uptake of triglycerides and cholesterol (61). Recent studies have demonstrated that mice in which a Hu Apo E4 cDNA has been introduced into the second coding exon (a knock-in mouse) show elevated levels of cholesterol in serum and lower levels in brain as compared to mice expressing endogenous Apo E (62). Conditions that elevate cholesterol *in vivo*, lead to accelerated deposition of Aβ in transgenic mice expressing Hu APPswe (63), whereas lowering levels of cholestrol reduces the levels of Aβ1–40 and Aβ1–42 (64). Changes in cholesterol levels in non-neuronal cells also lead to alterations in the secretion of Aβ peptides (65,66). Lowering cholesterol levels stimulates proteolytic cleavage of APP in a manner that precludes the secretion of Aβ1–40 and Aβ1–42 (67). The mechanisms by which these changes in Aβ levels occur are unclear, but recent studies have suggested that APP may reside in novel cholesterol-rich domains in the lipid membrane (68,69). Hence changes in the subcellular location or maturation of APP may lead to alterations in proteolytic processing. In addition, cholesterol may directly impact on the rate of Aβ aggregation (70). Collectively, these studies all point to cholesterol levels as a key modulator of Aβ production and perhaps aggregation.

We would argue that if changes in cholesterol metabolism are the basis for increased risk for AD in individuals with the E4 allele, then the hyper-expression of Apo E would do little to hasten the rate of Aβ deposition because other factors may limit the impact of the increased Apo E4 protein levels on cholesterol metabolism. For example, the Apo E receptors may already be saturated or the availability of the appropriate lipid components with which Apo E associates may be in limiting concentrations. Thus, hyper-expression may not equate to hyper-function.

We also note that Apo E may influence Aβ production and deposition through interactions with LRP (low density lipoprotein receptor-related-protein). LRP is a major receptor for Apo E in brain (71,72), and it is possible that Apo E exerts its effects on Aβ deposition through LRP signaling. One of the principal phenotypes of Apo E knockout mice that express mutant APP is a dramatic reduction in the number of neuritic deposits (31,33). Intriguingly, the binding of Apo E to LRP increases neurite extension in cultured hippocampal neurons (71). Alternatively, LRP has been shown to interact directly with APP and Aβ to modulate the metabolism of β-peptides

(73–76). These interactions may be modulated by Apo E as it competes for this receptor. Thus, there is reason to suspect that the mechanisms by which Apo E influences Aβ deposition may involve the activity of LRP. In this scenario, the hyper-expression of Apo E4 may simply saturate the receptor so that hyper-function is not achieved, and hence there is no proportionality between the levels of Apo E4 expression and the rate of Aβ deposition.

Apo E4 toxicity and its impact on cytoskeletal pathology

It has been suggested that Apo E4 may be directly toxic to neurons (34,77,78) or may modulate the toxicity of Aβ (58,79). In a study of twins, individuals with ApoE4 alleles appeared to have smaller hippocampal formations (78). Fragments of Apo E4, derived by thrombin cleavage, were shown to be toxic to primary neuronal cultures (34). However, obvious neurodegeneration was absent in our Apo E4 mice.

It has also been suggested that Apo E4 may impact the metabolism of tau (80). A recent study of FVB/N mice that express Hu Apo E4, under the transcriptional control of the Hu Thy-1 promoter, reported increased phosphorylation of tau proteins in aged animals (>18 months) (46). However, despite the change in phosphorylation state, these animals did not develop neurofibrillary tangles or frank neurodegeneration. In our animals, we did not observe a marked change in the levels of tau phosphorylation nor did we observe a change in the aggregation state of tau. Whether the differences between our study and those of Tesseur *et al.* (46) arise from differing responses of the host strain of mice to Apo E overexpression is uncertain.

Neuronal versus astrocytic expression of Apo E

Recent reports have suggested that, in humans, CNS neurons express Apo E, albeit at much lower levels than astrocytes (61,81–88), and it has been hypothesized that this neuronal expression may disproportionately impact on the pathogenesis of AD (89). Our study demonstrates that the hyper-expression of Hu Apo E4 in neurons does not disproportionately modulate Aβ deposition or tau metabolism. We suggest that our study provides a rigorous test of whether neuronally expressed Apo E4 can impact on Aβ deposition and other AD-related pathologies in mice, resulting in a negative outcome.

CONCLUSIONS

Our study provides the first examination of the influence of Apo E, expressed at levels several fold higher than normal, on the age of appearance, relative load, distribution and character of Aβ deposits in mice that express Mo/Hu APPswe or co-express Mo/Hu APPswe with PS1dE9. According to our measurements, the levels of Hu Apo E4 secreted by CNS astrocytes would be at least five times the normal level of mouse Apo E. In this setting, we observe no proportional acceleration in Aβ deposition nor do we observe a proportional alteration in tau metabolism. We interpret these findings as a demonstration that Apo E4 polypeptides, alone, probably do not possess activities that directly impact on the aggregation state of Aβ or tau metabolism. Instead, we argue that the mechanisms by which Apo E influences Aβ aggregation involves an activity that cannot be augmented by hyper-expression, ruling out direct protein–peptide interaction as a mechanism. We suggest that the increased risk for AD that is associated with Apo E4 alleles may reflect subtle differences in the functional activity (binding to lipid particles or receptor interactions) of the different allelic variants. An alternative explanation would posit that perhaps these Apo E alleles are in disequilibrium with another locus and it is the 'other' locus that actually specifies risk for AD.

MATERIALS AND METHODS

Transgenic animals

Mice expressing Mo/Hu APPswe and PS1dE9 have been previously described (10,14,90). Mice expressing Hu Apo E4 were generated as follows. A Hu ApoE4 cDNA was generated by a three-way PCR strategy that utilized Hu ApoE3 cDNA as template (pJS382) and changed the cystine at codon 112 to arginine (to create the Apo E4 genotype) while destroying a *Not*I site within the open reading frame of the cDNA (allowing the linearization of the MoPrP transgenic vector using a *Not*I digest before microinjection). The primers used were: 5′-sense-primer CGCCCGGTCGACCACAGGCAGGAAGATGAAGG-TTCTG; middle primer CTGCACCAGGCGGCCTCTCACGT-CCTCCATG; and 3′-antisense-primer CGCCCGGTCGACGG-GGTGGCGTGGGGTCGCATG. The sense and antisense primers encode *Sal*1 restriction sites at their termini to facilitate cloning into the MoPrP.Xho vector (35). The PCR product was gel-purified and ligated into the TA cloning vector (Invitrogen, Carlsbad, CA). Clones containing full-length Hu ApoE4 cD-A without the *Not*I site were identified and verified by DNA sequence analysis. The cDNA insert was excised by *Sal*1-digestion cloned into the *Xho*1 site of MoPrP.Xho. The orientation of the insert was verified by restriction endonuclease digestion and PCR analysis. Plasmids containing a single Apo E4 cDNA insert were amplified, purified, linearized by *Not*1 digestion, and gel purified for microinjection as previously described (35).

Analysis of Apo E4 transgene expression

Mouse hemibrains were homogenized in phosphate-buffered saline (PBS) containing protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin A) using a polytron tissue homogenizer. For soluble and insoluble protein fractions, homogenates were centrifuged at 200 000 *g* for 30 min. Protein concentrations of each homogenate were determined by protein assay (Pierce, Rockford, IL). The homogenates were diluted further in 2× Laemmli sample buffer (91) and sonicated for 15 s. Soluble and insoluble proteins were resolved on gradient SDS–polyacrylamide gel (4–20%) and transferred to nitrocellulose filter membranes. Nitrocellulose membranes were blocked in 5% non-fat dry milk in PBS. After an overnight incubation with the primary antibodies in PBS supplemented with 5% (w/v) non-fat dry milk and 0.05% (v/v) Tween-20, the blots were incubated with an anti-mouse IgG secondary antibody conjugated to peroxidase and developed with enhanced chemiluminescence (Amersham, Piscataway, NJ). The following antibodies were used: goat anti-Hu Apo E (Calbiochem, San Diego, CA), mouse monoclonal anti-Hu

Apo E (Boehringer Mannheim, Indianapolis, IN) and mouse monoclonal anti-Mo Apo E (Biodesign International, ME).

Generation of GST–Mo Apo E fusion proteins

Total RNA was isolated with Trizol (Life Technologies, Grand Island, NY) from total adult mouse brain. Reverse transcription was performed in 30 µl 1× RT buffer (Life Technologies) using 2 µg of total RNA, 0.08 U $pd(T)_{12–18}$ oligo (Pharmacia, Piscataway, NJ), 0.2 mM deoxyribonucleotide triphosphates (Pharmacia), 10 mM dithiothreitol (Life Technologies) and 200 U of Superscript II (Life Technologies). Single-stranded cDNA was synthesized during 1 h of incubation at 37°C. Reverse-transcribed RNA (1 µl) was added to a PCR reaction mixture containing forward primer for ApoE designed with a *Bam*H1 restriction site at the 5′ end: 5′-GGATCCATGAAG-GCTCTGTGGGCCGTGC-3′ and reverse primer for ApoE with a *Sal*1 restriction site at the 5′ end: 5′-GTCGACTCATT-GATTCTCCTGGGCCAC-3′ (1 µM each), 0.5 mM deoxyribonucleotide triphosphates, 1.5 mM $MgCl_2$ and *Taq* DNA polymerase (1 U; Life Technologies, Bethesda, MD) in a total volume of 50 µl. Amplification cycles as follows: 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, repeated 30 times. The PCR fragment generated was cloned into pCR II (Invitrogen, San Diego, CA) and subcloned into an expression vector pGEX-4T-2 between the restriction sites *Bam*H1–*Sal*1 (Promega). *Escherichia coli* were transformed with pGEX-4T-2-mouse ApoE plasmid. Overnight cultures in LB medium of *E.coli* transformed with pGEX-4T-2-mouse Apo E were diluted 1:10 in 10 ml of fresh LB medium and grown for 2 h at 37°C before adding IPTG to 0.1 mM. After a further 3 h, cells were sedimented by centrifugation then resuspended in Laemmli buffer. A serial dilution of bacterial protein extract containing GST–Mo Apo E fusion protein and a serial dilution of bovine serum albumin protein (BSA) were separated on 4–20% SDS–polyacrylamide gel and stained with Coomassie blue. The quantity of the GST–Mo Apo E fusion protein in the bacterial extract was estimated relative to BSA.

Primary neuronal and glial cultures

Cerebral cortices were dissected from postnatal day 1 mice and dissociated by treatment with 0.25% trypsin (Life Technologies) followed by trituration with a fire-polished Pasteur pipette. For western blot analysis, 10^6 cells were plated onto 35 mm tissue culture dishes. Tissue culture dishes were coated with 33 µg/ml poly-D-lysine. The cells were plated in Neurobasal medium (Life Technologies) supplemented with B27 (Life Technologies), 2 mM Glutamax (Life Technologies), 25 µM β-mercaptoethanol and streptomycin/amphotericin B (Life Technologies). Three days after plating, 50% of the medium was changed and subsequently changed every 6 days.

For glial cultures, the dissociated cells were plated in DMEM (Life Technologies) supplemented with 10% (v/v) fetal calf serum (Life Technologies). At days 3 and 10, cells were treated for 1 h with 100 mM L-glutamate to kill neurons.

At day 15 *in vitro*, the culture medium was replaced with fresh Neurobasal medium for neurons, as described above or DMEM supplemented with G5 supplement (Life Technologies) for glial cells. After 2 days, all culture media from three wells (35 mm) were collected and pooled. Lysates of cells were prepared by extraction in Tris-buffered saline with 1 mM EDTA, 2% SDS, 1% NP40, 0.5% deoxycholate and a protease inhibitor cocktail (Sigma, St Louis, MO). Lysates from three wells were pooled before analysis by SDS–PAGE and immunoblot.

Aβ peptide determination

Hemibrains were homogenized using a Dounce tissue grinder in 24 volumes of 1% CHAPS/PBS supplemented with protease inhibitors (1 mM PMSF, 5 µg/ml leupeptin, 30 µg/ml aprotinin and 1 µM pepstatin). After 30 min of incubation at 4°C on a tube rotator, the homogenates were centrifuged at 100 000 *g* for 1 h. Supernatants were immunoprecipitated with the monoclonal antibody 26D6 (specific for Aβ1–12) followed by electrophoretic separation of Aβ peptides with bicine/urea SDS–PAGE (92). Proteins were transferred to PVDF membranes and blotted with 26D6 conjugated to horseradish peroxidase. Aβ peptides were visualized using ECL-Plus (Amersham) and quantified by scanning laser densitometry. Amounts (ng) of Aβ1–40 and Aβ1–42 were estimated by comparing the band densities of extracts to the band densities of Aβ standards.

Histology and immunocytochemistry

Tissues used in histological analyses were prepared in either of two ways. For aged Apo E4 transgenic mice, animals were deeply anesthetized in metofane, then perfused transcardially with 4% paraformaldehyde in PBS. The brains were removed and placed in 10 volumes of 4% paraformaldehyde in PBS at 4°C. After 24 h, the brains were placed in 10 volumes of PBS and stored at 4°C until processed for sectioning. In analyses of APPswe/Apo E4 and APPswe/PS1dE9/Apo E4 mice, we relied on immersion fixed tissues. Animals were deeply anesthetized in metofane, decapitated and the brains were removed. One hemi-brain (sagittal bisection) was immediately frozen on dry-ice for biochemical studies. The other hemibrain was placed in 10 volumes of 4% paraformaldehyde in PBS at 4°C. After 48 h, the brains were placed in 10 volumes of PBS and stored at 4°C until processed for sectioning.

Processing for histology and immunocytochemistry to detect Aβ deposits and Aβ1–40 and Aβ1–42 followed previously described standard procedures (14). Briefly, prior to immunostaining, paraffin-imbedded sections (supported on Vectabond-coated slides) were deparaffinized by oven heating, followed by a 3 min incubation in 70% formic acid (used in immunostains of Aβ only), further deparaffinization in xylene followed by washes in 100% ethanol, 95% ethanol, 70% ethanol and water. Endogenous peroxidase activities were quenched by 30 min incubations in 3% H_2O_2 (in methanol), before sections were microwaved for 5–7 min in water, cooled for 5 min, rinsed with water, then washed in Tris-buffered saline (TBS; 0.05 M Tris–HCl pH 7.6 and 0.25 M NaCl). Non-specific epitopes were then blocked for 1 h with 3% normal goat serum and 0.1% Triton X-100 in TBS. Primary antibodies, which included rabbit anti-Hu Aβ40 and Aβ42 (Quality Control Biochemicals, Hopkinton, MA), mouse monoclonal anti-Hu Apo E (Boehringer Mannheim), and rabbit anti-Hu presenilin 1 [C-terminal fragment–PS1Loop antiserum (93,94)] were diluted in TBS with 2% normal goat serum and incubated overnight at 4°C in a humid chamber. Sections were then washed in TBS three times, for 5 min each,

and incubated with secondary antibodies and peroxidase-coupled streptavidin as described by the manufacturer (Vectastain ABC Kit; Vector Laboratories Inc., Burlingame, CA). All sections were lightly counter-stained with hematoxylin and eosin, following standard histological procedures.

Immunoblot analysis of tau phosphorylation

Mouse cerebral cortex, hippocampus, etc., were dissected and sonicated in 1 ml of PBS, pH 7.4. The protein concentration was determined by the BCA method (Pierce). To 50 µl of homogenate was added 1 µl 100× protease inhibitor cocktail (Sigma), 4 µl of 25× phosphatase inhibitors (containing 500 mM NaF, 25 mM Na_3VO_4, 125 mM $Na_4P_2O_7$), 1 µl of 10 µM okadaic acid, followed by 50 µl of 2× Laemmli sample buffer (91). The samples were boiled for 10 min, resolved on 10% SDS–polyacrylamide gel, then transferred for immunoblot as described above. PHF-1 (1:200; a generous gift form Dr Peter Davies, Albert Einstein College of Medicine, NY) was used in immunoblotting.

Protein dot filter

Homogenates prepared as above were centrifuged at 2000 *g* for 5 min at 4°C in a microcentrifuge. The supernatant was aliquoted and frozen at –70°C until used. Protein concentrations were determined by BCA. Before filtering, the samples were thawed and diluted with PBS containing 1% SDS. Filters were cellulose acetate membranes—0.2 µm pore size (OE66; Schleicher & Schuell, Keene, NH). Diluted samples (~100–200 µl) were filtered using a 96-well dot-blot apparatus (Bio-Rad Laboratories, Hercules, CA) then washed twice with PBS (pH 7.4), 500 µl each. Proteins trapped by the filter were detected by immunostaining following protocols used in immunoblotting (14).

Neurochemistry

ChAT activities were measured following previously published protocols (95). Briefly, to the brain homogenates made above, add 10% Triton X-100 to 0.5% final. Reaction mixtures [0.05 M sodium phosphate buffer, pH 7.4, 0.3 M NaCl, 0.02 M EDTA, 8 mM choline chloride (freshly made), 0.1 mM physostigmine (freshly made)] were generated and supplemented with ^{3}H acetyl coenzyme A (Amersham) equivalent to 10 000 c.p.m. per 100 µl (~6 µl in 10 ml of buffer). Scintillation tubes with 100 µl reaction buffer were held on ice until the addition of 10 µl of diluted tissue homogenate (for cortex, dilute ~5–10×, for hippocampus, dilute ~2–5× in homogenization buffer). After 15 min incubation in water at room temperature, the reactions were stopped with 1 ml of 0.001 M sodium phosphate buffer, pH 7.4. Four milliliters of non-aqueous scintillation fluid, containing 0.1% kalignost, was added and the mixture was shaken vigorously. Acetylcholine separates into the organic phase of the scintillation fluid and is detected in a scintillation counter. Values were compared to a standard curve generated from ChAT from Sigma.

AChE activities were measured following previously published protocols (96). The homogenates described above (in 0.5% Triton X-100), were diluted further with 10 volumes of 0.1 M sodium phosphorylated buffer containing 10 mM $MgCl_2$, pH 7.0, then centrifuged at 12 000 *g* for 10 min at 4°C. A 50 µl aliquot of the supernatant was diluted further with 1 ml of 0.1 M phosphate buffer, pH 8.0, before 30 µl of 0.01 M 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (Sigma) in PBS and 20 µl of 25 mM acetylthiocholine iodide (Sigma) were added. Absorbance (412 nm) was recorded at intervals of 15 s beginning after a 200 s delay, using a Ultrospec 3000 spectrophotometer (Pharmacia). The reactions were monitored over 900 s, in eight parallel cells with each sample duplicated.

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