The Journal of Neuroscience, December 15, 2002, 22(24):10539–10548

Modulation of Alzheimer-Like Synaptic and Cholinergic Deficits in Transgenic Mice by Human Apolipoprotein E Depends on Isoform, Aging, and Overexpression of Amyloid β Peptides But Not on Plaque Formation

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The most frequent human apolipoprotein (apo) E isoforms, E3 and E4, differentially affect Alzheimer's disease (AD) risk (E4 > E3) and age of onset (E4 < E3). Compared with apoE3, apoE4 promotes the cerebral deposition of amyloid β (Aβ) peptides, which are derived from the amyloid precursor protein (APP) and play a central role in AD. However, it is uncertain whether Aβ deposition into plaques is the main mechanism by which apoE isoforms affect AD. We analyzed murine apoE-deficient transgenic mice expressing in their brains human APP (hAPP) and Aβ together with apoE3 or apoE4. Because cognitive decline in AD correlates better with decreases in synaptophysin-immunoreactive presynaptic terminals, choline acetyltransferase (ChAT) activity, and ChAT-positive fibers than with plaque load, we compared these parameters in hAPP/apoE3 and hAPP/apoE4 mice and singly transgenic controls at 6–7, 12–15, and 19–24 months of age. Brain aging in the context of high levels of nondeposited human Aβ resulted in progressive synaptic/cholinergic deficits. ApoE3 delayed the synaptic deficits until old age, whereas apoE4 was not protective at any of the ages analyzed. Old hAPP/apoE4 mice had more plaques than old hAPP/apoE3 mice, but synaptic/cholinergic deficits preceded plaque formation in hAPP/apoE4 mice. Moreover, despite their different plaque loads, old hAPP/apoE4 and hAPP/apoE3 mice had comparable synaptic/cholinergic deficits, and these deficits were found not only in the hippocampus but also in the neocortex, which in most mice contained no plaques. Thus, apoE3, but not apoE4, delays age- and Aβ-dependent synaptic deficits through a plaque-independent mechanism. This difference could contribute to the differential effects of apoE isoforms on the risk and onset of AD.

Key words: acetylcholine; aging; Alzheimer's disease; amyloid; apolipoprotein E; cholinergic; neurodegeneration; synapses; transgenic

Alzheimer's disease (AD) is an age-dependent neurodegenerative disorder that is characterized by a progressive cognitive decline and by characteristic morphological CNS alterations, including deposition of amyloid β peptides (Aβ) in parenchymal plaques and cerebral blood vessels; intraneuronal formation of neurofibrillary tangles; and loss of neuronal subpopulations, synaptophysin-immunoreactive (SYN-IR) presynaptic terminals, and cholinergic fibers (Terry et al., 1999). Different gene products have been implicated in the pathogenesis of this disease. Early-onset autosomal dominant forms of familial AD (FAD) have been linked to mutations in genes encoding amyloid precursor protein (APP), presenilin 1, and presenilin 2 (Selkoe, 2001). Mutations in these genes alter the processing of APP such that increased amounts of either total Aβ or Aβ ending at residue 42 (Aβ42) are produced (Selkoe, 2001).

Although mutations in APP or presenilin genes account for only a fraction of AD cases, inheritance of the apolipoprotein (apo) E ε4 allele is the major known genetic risk factor for the most common type of AD (Farrer et al., 1997). ApoE is a 34 kDa lipid carrier protein that participates in the maintenance and repair of neurons (Mahley and Huang, 1999). It is expressed at high levels in the brain and can be produced by diverse cell types, including neurons, astrocytes, and microglia (Boyles et al., 1985; Stone et al., 1997; Buttini et al., 1999; Xu et al., 1999, 2000; Dekroon and Armati, 2001). In humans, apoE occurs in three major isoforms, which are associated with different risks of developing AD (E4 > E3 > E2) (Corder et al., 1993; Farrer et al., 1997). The two most frequent isoforms differ not only in their effects on Aβ deposition (E4 > E3) (Rebeck et al., 1993; Schmechel et al., 1993; Berr et al., 1994; Heinonen et al., 1995; Hyman et al., 1995; Gearing et al., 1996; Ishii et al., 1997; Johnson et al., 1998; McNamara et al., 1998; Holtzman et al., 2000a,b) but also in their capacity to protect the brain against diverse injuries, including those elicited by excitotoxins, ischemia, and trauma (E3 > E4) (Sheng et al., 1998; Buttini et al., 1999, 2000; Horsburgh et al., 2000; Sabo et al., 2000). Although any or all of these effects might play a role in AD, some studies have suggested that the main effect of apoE isoforms is through plaque formation (Holtzman et al., 2000a,b), whereas others have provided evidence for plaque-independent mechanisms (Raber et al., 2000).

Received April 17, 2002; revised Aug. 29, 2002; accepted Sept. 4, 2002.

This work was supported by National Institutes of Health Grants AG11385 and NS41787 and by a Zenith Award (99-1847) from the Alzheimer's Association. We thank D. Selkoe for the 1282 antibody, J. Carroll and S. Gonzales for preparation of graphics, G. Howard and S. Ordway for editorial assistance, and D. McPherson for administrative assistance.

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Differentiating whether AD-related neuronal deficits are caused by plaques, Aβ fibrils, or nonfibrillar Aβ species is an important conundrum in AD research (Klein et al., 2001). Human Aβ is neurotoxic when added to cultures of neural cells or tissue sections (Pike et al., 1993; Yankner, 1996; Klein et al., 2001), injected into the brain (Geula et al., 1998), or produced in neurons of transgenic mice (Games et al., 1995; Masliah et al., 1996, 2001; Nalbantoglu et al., 1997; Calhoun et al., 1998; Price et al., 1998; Hsia et al., 1999; Mucke et al., 2000). However, both *in vitro* and *in vivo*, Aβ can exist in diverse conformational states. Which of these states is responsible for the dysfunction and degeneration of neurons in AD remains a matter of active study and debate (Terry, 1996; Davis and Chisholm, 1997; Hartley et al., 1999; Hsia et al., 1999; Lue et al., 1999; McLean et al., 1999; Mucke et al., 2000; Näslund et al., 2000; Klein et al., 2001). Both deposited and nondeposited forms of Aβ might contribute to the pathogenesis of AD, but their relative contributions have been difficult to dissect, both in humans and in experimental models. Although neuritic dystrophy appears to be closely linked to plaques (Knowles et al., 1999), evidence is mounting that AD-related synaptic degeneration and functional neuronal impairments may be caused primarily by nondeposited forms of Aβ (Lambert et al., 1998; Hartley et al., 1999; Holcomb et al., 1999; Hsia et al., 1999; Lue et al., 1999; McLean et al., 1999; Mucke et al., 2000; Raber et al., 2000; Klein et al., 2001).

To investigate the effects of apoE isoforms on AD-related deficits *in vivo* and, in particular, the possible relationship between the development of such deficits and the amyloidogenic effect of apoE4, we analyzed transgenic mice expressing human APP (hAPP)/Aβ in combination with either apoE3 or apoE4 in the brain. We focused our analysis on SYN-IR presynaptic terminals, choline acetyltransferase (ChAT) activity, and cholinergic fibers, because decreases in these parameters correlate well with cognitive decline in AD (Terry et al., 1991; Samuel et al., 1997; Sze et al., 1997; Brown et al., 1998). ChAT synthesizes the neurotransmitter acetylcholine (ACh) and is produced by cholinergic neurons clustered in a number of cell groups in the basal forebrain (Cummings, 2000). Its activity is 40–90% lower in AD brains than in control brains, and this reduction correlates with cognitive decline (Perry et al., 1978; Davies, 1979; Wilcock et al., 1982; Sims et al., 1983; Neary et al., 1986; Bierer et al., 1995; Samuel et al., 1997; Cummings, 2000).

In mice, lack of murine apoE or expression of human Aβ in the presence of murine apoE is also associated with age-dependent synaptic and cholinergic deficits (Games et al., 1995; Gordon et al., 1995; Masliah et al., 1995a,b, 1996, 2001; Buttini et al., 1999, 2000; Hsia et al., 1999; Mucke et al., 2000). However, these models did not allow an analysis of potential interactions between human Aβ and human apoE. In the current study, we analyzed hAPP/apoE3 and hAPP/apoE4 mice that express human Aβ in the context of human apoE and therefore more closely simulate the situation encountered in the human brain.

Previous studies of hAPP/apoE transgenic mice focused primarily on amyloid deposition and plaque-related pathology (Holtzman et al., 1999, 2000a,b) but did not examine effects of human apoE isoforms on other AD-related deficits, such as loss of SYN-IR presynaptic terminals, ChAT activity, or cholinergic fibers. The results we obtained in the current study suggest that apoE3 but not apoE4 can delay some age- and Aβ-dependent neuronal deficits through a plaque-independent neuroprotective mechanism.

Table 1. Relative levels of SYN-IR presynaptic terminals in transgenic mice

	Age		
Genotype[a]	6–7 months	12–15 months	19–24 months
Neocortex			
apoE3	+++	+++	+++
apoE4	++	+	+
hAPP/apoE3	+++	++(+)	+
hAPP/apoE4	++	+	+
hAPP	+	+	+
Apoe$^{-/-}$	+	+	+
Hippocampus			
apoE3	+++	+++	+++
apoE4	+++	+	+
hAPP/apoE3	+++	+++	+
hAPP/apoE4	+++	+	+
hAPP	+++	+	+
Apoe$^{-/-}$	+++	+	+

[a]All mice (n = 4–7 per genotype and age group) lacked murine apoE. +++, Levels comparable with those found in nontransgenic wild-type mice (Buttini et al., 1999); ++, moderate reduction; +, most prominent reduction. For quantitative data, see Figure 2 and Results. For illustrations, see Figure 4.

MATERIALS AND METHODS

Animals. The generation of murine apoE-deficient transgenic mice expressing FAD-mutant hAPP, directed by the platelet-derived growth factor (PDGF) B chain promoter (line J9), in combination with either apoE3 or apoE4, directed by the neuron-specific enolase (NSE) promoter, has been described previously (Raber et al., 1998, 2000; Buttini et al., 1999; Hsia et al., 1999; Mucke et al., 2000). For the current study, we used a total of 102 mice, bred to be >95% C57BL/6J. Six genotypes and three age groups were analyzed (Table 1). Each genotype and age group contained approximately the same number of males and females. No significant differences were identified between age- and genotype-matched male and female mice with respect to any of the endpoints analyzed. Mice were anesthetized with chloral hydrate and flush-perfused transcardially with 0.9% saline. Brains were removed and divided sagitally. One hemibrain was postfixed in phosphate-buffered 4% paraformaldehyde, pH 7.4, at 4°C for 48 hr for vibratome sectioning. The neocortex, hippocampus, and medial septum were dissected from the other hemibrain on ice, frozen immediately on dry ice, and stored at −70°C until analysis.

Aβ measurements. Frozen brain tissues were homogenized in guanidine buffer, and human Aβ peptides were quantitated by ELISA as described previously (Johnson-Wood et al., 1997). The Aβ1–42 ELISA detects only Aβ1–42, whereas the Aβ1-x ELISA detects Aβ1–40, Aβ1–42, Aβ1–43, and C-terminally truncated forms of Aβ containing amino acids 1–28.

Immunohistochemistry for Aβ was performed on 50 μm free-floating vibratome sections of paraformaldehyde-fixed brain tissue. Endogenous peroxidase activity was quenched by incubating sections in 3% H_2O_2 in PBS with 0.5% Triton X-100 for 20 min. To block nonspecific binding sites, sections were incubated for 1 hr at room temperature in 15% goat serum (Vector Laboratories, Burlingame, CA). Sections were then incubated overnight at 4°C with a 1:6000 dilution of R1282 antibody (gift from D. Selkoe, Brigham and Women's Hospital, Boston, MA). Sections were then washed twice in PBS and incubated for 1 hr with a 1:200 dilution of biotin-labeled anti-rabbit as a secondary antibody (Vector Laboratories). Secondary antibody binding was detected with the ABC Elite kit (Vector Laboratories) using diaminobenzidine and H_2O_2 as chromagenic substrates. Aβ-immunoreactive deposits were visualized by bright-field microscopy with a 4× objective and photographed with an Axiocam digital camera (Zeiss, Weimar, Germany).

Although the Aβ antibody detects both diffuse and fibrillar amyloid deposits, thioflavin-S (thio-S) detects primarily fibrillar amyloid and, hence, is widely used to detect more mature amyloid plaques (Schmidt et al., 1995). Thio-S-positive amyloid plaques were visualized in 50 μm vibratome sections with a staining protocol adapted from Schmidt et al.

(1995). Briefly, sections were mounted onto SuperFrost glass slides (Fisher Scientific, Houston, TX) and air-dried. After fixation for 10 min in 3.7% formalin, they were rinsed twice in PBS and immersed in 0.25% $KMnO_4$ in PBS for 10 min. After another rise in PBS, they were immersed for 5 min in a solution containing 2% $K_2O_5S_2$ and 1% oxalic acid. After a 10 min rinse in water, sections were incubated for 10 min in a 0.015% thio-S solution in 50% ethanol, differentiated in 50% ethanol, washed in water, air-dried, and coverslipped. Sections were examined by confocal microscopy (MRC 1024 or Radiance 2000; Bio-Rad, Hercules, CA) with an FITC filter set. Digitized images were transferred to a computer, and the average percentage area of the hippocampus occupied by thio-S-positive plaques in three to four sections per mouse (plaque load) was determined with NIH Image.

ApoE measurements. ApoE levels in brain tissues were quantitated by Western blot analysis as described previously (Huang et al., 1996, 2001). Briefly, brain tissues were homogenized in 0.3 ml of ice-cold lysis buffer containing 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 4% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, and protease inhibitors. After determination of protein concentrations by the Lowry method, samples (100 μg total proteins per lane) were subjected to SDS-PAGE and analyzed by Western blotting with an anti-human apoE antibody. Sample bands were compared with standards containing different amounts of recombinant human apoE3 or apoE4 by densitometric analysis.

ApoE immunohistochemistry was performed on 50 μm vibratome sections. Endogenous peroxidase was quenched by incubation in 3% H_2O_2/10% methanol in PBS for 15 min, and nonspecific binding sites were blocked with a mixture of 10% rabbit serum, 1% nonfat dry milk, 0.2% gelatin, and 0.2% Triton X-100. Anti-apoE (Calbiochem, San Diego, CA) was diluted 1:28,000, and biotin-labeled anti-goat secondary antibody was diluted 1:200. Secondary antibody binding was detected with the ABC Elite kit (Vector Laboratories) using diaminobenzidine and H_2O_2 as chromagenic substrates. The specificity of this immunostain has been documented previously (Buttini et al., 1999). Photomicrographs were taken with an AxioCam digital camera (Zeiss) coupled to an Olympus Optical (Tokyo, Japan) BX-60 microscope.

SYN-IR presynaptic terminals. Vibratome sections ($n = 2$ per mouse) labeled first with an anti-synaptophysin monoclonal antibody (diluted 1:40; Boehringer Mannheim, Mannheim, Germany) and then with an FITC-coupled anti-mouse antibody (diluted 1:75; Vector Laboratories) were imaged, essentially as described previously (Buttini et al., 1999), with a Radiance 2000 laser scanning confocal microscope mounted on an Olympus BX-60 microscope using a 60× oil objective. Sections were assigned code numbers to ensure objective assessment, and codes were not broken until the analysis was complete. The density of SYN-IR presynaptic terminals was assessed in the strata radiatum and lacunosum of the hippocampus (CA1 subfield) and in layers 2–5 of the frontoparietal neocortex. For each mouse, we analyzed three to four optical sections of the stratum radiatum and the stratum lacunosum and four to six optical sections of the neocortex.

Staining of sections with antibody dilutions that are either too high or too low can obscure differences between experimental and control groups, yielding false negative results. To avoid this problem, we injected wild-type mice with saline or kainate as described previously (Buttini et al., 1999) and performed pilot experiments to determine the dilutions of primary and secondary antibodies at which differences in the intensity of synaptophysin immunostaining between these groups were maximal. The optimized antibody dilutions were then used for the current study as described above. To further ensure the reliability of our quantitations, for each experiment, we first determined the linear range of fluorescence intensity and density of SYN-IR presynaptic terminals in sections from wild-type mice (data not shown). This setting of the confocal microscope was then used to collect all images analyzed in the same experiment. The density of SYN-IR presynaptic terminals was expressed as percentage of the image area occupied by immunoreactive structures of defined signal intensity. Digitized images were transferred to a computer (Apple Computers, Cupertino, CA) and analyzed with the public domain program NIH Image. As reviewed recently (Buttini et al., 1999), the above approach has been used successfully in various experimental models of neurodegeneration and diseased human brain and has been validated by comparisons with quantitative immunoblots, ELISAs, and the optical disector probe.

ChAT activity and immunoreactivity. ChAT activity in the medial septum of 3–10 mice per genotype was determined by previously described procedures (Yeo et al., 1997). Medial septum tissue was sonicated in 50 mM Tris/0.2% Triton X-100 buffer, pH 7.4 (diluted 1:20, wet w/v), and centrifuged at maximum speed in an Eppendorf centrifuge. The supernatant was transferred to another tube, and soluble protein levels were determined by bicinchoninic acid assay (Pierce, Rockford, IL). Samples from each mouse were diluted 1:5 with 50 mM Tris/0.5 M sodium-phosphate buffer, pH 7.0, to a final volume of 50 μl, mixed with 50 μl of reaction buffer (0.4 M NaCl, 80 μg/ml eserine, 12 mM choline chloride, 10 μg/ml albumin, and 0.05 μCi ^{14}C-labeled acetyl CoA in Na-phosphate buffer), and incubated at 37°C for 30 min. Background readings for each mouse were obtained by boiling duplicate tissue samples for 5 min before mixing them with the reaction buffer. Reactions were stopped with 500 μl of ice-cold H_2O and loaded onto a column with 1 inch of Dowex 1X-A beads (Bio-Rad). Columns were washed twice with 600 μl of H_2O, and all of the column effluents were collected and counted on a Beckman (Fullerton, CA) scintillation counter. The amount of ACh synthesized was calculated and expressed as nanomoles per milligram of protein per hour.

Immunohistochemistry for ChAT was performed on 50 μm free-floating vibratome sections of paraformaldehyde-fixed brain tissue. Endogenous peroxidase activity was quenched by incubating sections in 3% H_2O_2 in PBS with 0.5% Triton X-100 for 20 min. To block nonspecific antibody binding, sections were incubated for 15 min in Superblock (Scytek, Logan, UT). Sections were then incubated overnight at 4°C with anti-ChAT antibody (diluted 1:3500; Chemicon, Temecula, CA). Sections were then washed twice in PBS and incubated for 1 hr with biotin-labeled anti-goat secondary antibody (diluted 1:200; Vector Laboratories). Secondary antibody binding was detected with the ABC Elite kit using diaminobenzidine and H_2O_2 as chromagenic substrates. For all sections, the development reaction was stopped after 2 min by transferring the sections into 0.1 M Tris, pH 7.5. After two washes in the same buffer, sections were mounted, air-dried, and coverslipped. Digitized images of the immunostained sections were obtained with a DEI-450 Optronics digital camera (Coleta, CA) mounted on a BX-60 microscope (Olympus Optical) using a 20× magnification lens, and the integrated optical density of the immunoperoxidase product over defined areas was quantitated with the BioQuant Image Analysis package (R& M Biometrics, Nashville, TN). For each mouse, three to four measurements per brain region were obtained and averaged. Four to seven animals were analyzed per group. Dilutions of primary and secondary antibodies and development times for the chromagenic reaction were optimized in pilot experiments to maximize the reliability of the immunostain (see also above). To further standardize our measurements, control sections from the same three wild-type mice (two sections per mouse) were included in each staining experiment, and all sections were processed and measured under similar conditions. Measurements in experimental mice were expressed as the percentage of integrated optical density readings obtained in corresponding regions of the wild-type control sections.

Statistical analyses. Statistical analyses were performed with the StatView 5.0 program (SAS Institute, Cary, NC). Differences among means were assessed by Mann–Whitney *U* test or by one-way ANOVA followed by Tukey–Kramer *post hoc* test as appropriate.

RESULTS

Generation of hAPP/apoE doubly transgenic mice lacking murine apoE

To study the influence of human apoE isoforms on AD-related neuropathological alterations, we bred murine apoE-deficient PDGF–hAPP transgenic mice from line J9 (Hsia et al., 1999; Mucke et al., 2000; Raber et al., 2000) (hAPP mice) with murine apoE-deficient NSE–apoE mice (apoE mice) that express apoE3 or apoE4 in the brain at levels similar to those in human cortex (Buttini et al., 1999). These crosses yielded six groups of mice, all of which lacked murine apoE (*Apoe*$^{-/-}$): doubly transgenic mice (hAPP/apoE3 or hAPP/apoE4), singly transgenic mice (hAPP, apoE3, or apoE4), and mice lacking human transgenes. ApoE3 and apoE4 singly transgenic mice show similar levels and distributions of human apoE in the brain (Raber et al., 1998; Buttini et al., 1999), and 6-month-old hAPP/apoE3 and hAPP/apoE4 mice have comparable levels of Aβ1–42 and Aβ1–x (approximates total Aβ) in the hippocampus (Raber et al., 2000). In the current study, we made similar observations (data not shown) and ex-

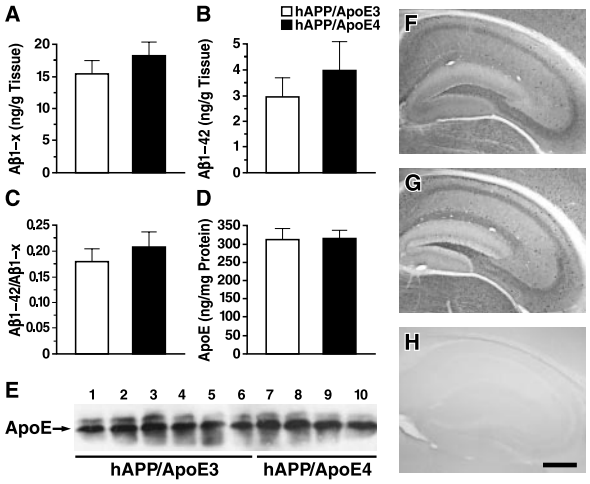

Figure 1. Comparable levels of human Aβ and apoE in brain tissues of hAPP/apoE3 and hAPP/apoE4 mice. *A–E*, Snap-frozen neocortex from 12- to 15-month-old hAPP/apoE3 mice and hAPP/apoE4 mice ($n = 4–7$ per genotype) was homogenized and analyzed for human Aβ1–x or Aβ1–42 by ELISA and for human apoE by quantitative Western blot analysis. hAPP/apoE3 (*open bars*) and hAPP/apoE4 (*filled bars*) mice had comparable Aβ1–x levels (*A*), Aβ1–42 levels (*B*), Aβ1–42/Aβ1–x ratios (*C*), and apoE levels (*D*). Values in *A–D* represent group means ± SEM. *E* depicts a representative Western blot demonstrating similar apoE levels in hAPP/apoE3 mice (*lanes 1–6*) and hAPP/apoE4 mice (*lanes 7–10*). *F–H*, The distribution of human apoE in the hippocampus of 12- to 15-month-old mice was determined by anti-apoE immunoperoxidase staining of paraformaldehyde-fixed vibratome sections in a hAPP/apoE3 mouse (*F*) and a hAPP/apoE4 mouse (*G*). An *Apoe*$^{-/-}$ mouse served as a control (*H*). Scale bar, 400 μm (applies to *F–H*).

tended these findings to another brain region and an older age group (Fig. 1).

ApoE3 but not apoE4 delays the age-dependent decline in SYN-IR presynaptic terminals in hAPP mice expressing high levels of soluble human Aβ

On the murine apoE wild-type background, hAPP mice develop an age-dependent loss of SYN-IR presynaptic terminals that is associated with major deficits in synaptic transmission strength (Hsia et al., 1999; Mucke et al., 2000). To detect potential apoE isoform-specific effects on age-related synaptic impairments in the context of human Aβ production, we determined the levels of SYN-IR presynaptic terminals in the neocortex (layers 2–5, frontoparietal region) and hippocampus (strata radiatum and lacunosum) by computer-assisted confocal image analysis. Six genotypes were analyzed at three age ranges (Table 1).

On the murine apoE-deficient background, hAPP mice also developed an age-related decline in SYN-IR presynaptic terminals in all three brain regions analyzed (Fig. 2). ApoE3 significantly delayed this decline, whereas apoE4 did not (Table 1, Figs. 2, 4). At 6–7 (neocortex) and 12–15 (neocortex and hippocampus) months of age, hAPP/apoE3 mice had higher levels of SYN-IR presynaptic terminals than hAPP/apoE4 mice, hAPP mice, or *Apoe*$^{-/-}$ mice lacking human transgenes (Table 1, Figs. 2, 4). The levels of SYN-IR presynaptic terminals in hAPP/apoE3 mice at these ages were normal (i.e., similar to those of age-matched wild-type controls without human transgenes) (Masliah et al., 1995b; Buttini et al., 1999, 2000; Hsia et al., 1999; Mucke et al., 2000) (data not shown). The levels of SYN-IR presynaptic terminals in hAPP/apoE4 mice were abnormally low and comparable to those in age-matched hAPP mice with or without murine

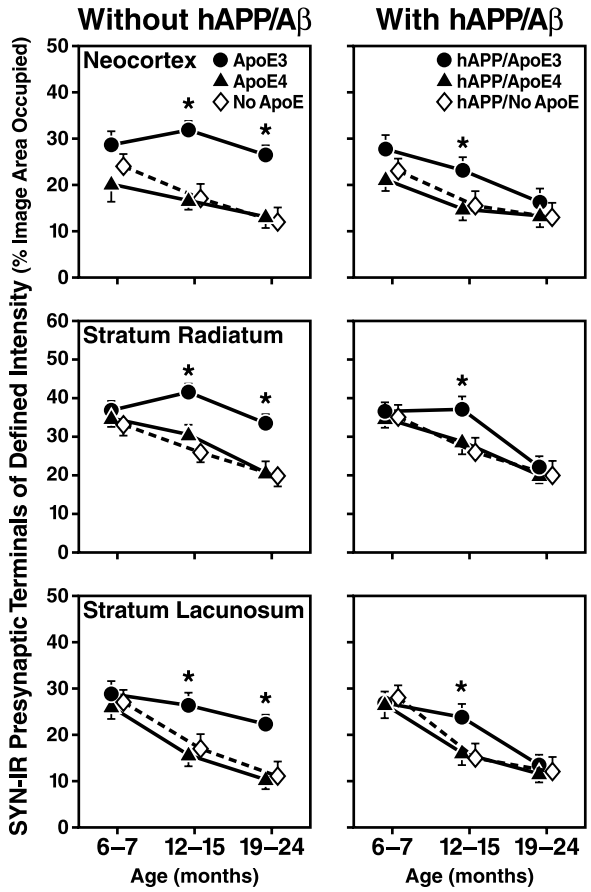

Figure 2. Age-related changes in presynaptic terminals of apoE singly transgenic (*left*) and hAPP/apoE doubly transgenic (*right*) mice lacking murine apoE. Littermates expressing neither human nor murine apoE served as additional controls. The density of SYN-IR presynaptic terminals in the strata radiatum and lacunosum of the hippocampus and in the neocortex was determined by confocal microscopy and computer-assisted image analysis. ApoE4 mice and hAPP/apoE4 mice showed a significant loss of SYN-IR presynaptic terminals at 12–15 and 19–24 months in all three brain regions. Like nontransgenic, wild-type (*Apoe*$^{+/+}$) mice (Buttini et al., 1999, 2000) (data not shown), apoE3 mice had no significant age-dependent loss of SYN-IR presynaptic terminals. In hAPP/apoE3 mice, the loss of SYN-IR presynaptic terminals was delayed until 19–24 months of age, when it was comparable to that in age-matched hAPP/apoE4 mice. ApoE-deficient mice with or without hAPP/Aβ expression developed an age-related loss of SYN-IR presynaptic terminals similar to that of apoE4 or hAPP/apoE4 mice. Results represent means ± SEM; $n = 4–7$ mice per genotype and age range; $^{*}p < 0.05$, age-matched mice expressing apoE3 versus apoE4 (Tukey–Kramer test).

apoE expression and to those in *Apoe*$^{-/-}$ mice lacking human transgenes (Fig. 2) (Masliah et al., 1995b; Buttini et al., 1999, 2000; Hsia et al., 1999; Mucke et al., 2000).

Although apoE3 was able to protect hAPP/apoE3 mice against synaptic deficits at 6–7 and 12–15 months of age, it failed to do so at 19–24 months of age (Table 1, Figs. 2, 4). By 19–24 months of age, levels of SYN-IR presynaptic terminals had declined to similar levels in hAPP/apoE3 and hAPP/apoE4 mice (Figs. 2, 4). In the absence of hAPP/Aβ, apoE3 was able to protect even the oldest murine apoE-deficient mice against synaptic deficits (Fig. 2). In contrast, apoE4 failed to protect against synaptic deficits at all ages tested, compared with apoE-deficient controls with or without hAPP/Aβ expression (Table 1, Fig. 2).

ApoE3 protects murine apoE-deficient mice against age-dependent cholinergic deficits but only if they do not produce human Aβ

To investigate the effect of apoE isoforms on ChAT in the presence or absence of human Aβ, we analyzed ChAT activity in the medial septum, which contains cell bodies of ACh-producing neurons, and ChAT-IR fibers in the neocortex and hippocampus, which contain cholinergic terminals. Murine apoE-deficient mice developed an age-dependent loss of ChAT activity and ChAT-IR fibers that was prevented by apoE3 but not by apoE4 (Fig. 3). However, this protective effect of apoE3 was not seen in mice expressing high levels of human Aβ, consistent with results obtained in hAPP mice expressing wild-type murine apoE (Boncristiano, 2002). Both hAPP/apoE4 and hAPP/apoE3 mice showed a comparable age-dependent loss of ChAT activity and ChAT-IR fibers (Figs. 3, 4).

The effects of apoE3 and apoE4 on synaptic deficits in hAPP/apoE mice are not attributable to their differential effects on plaque formation

To assess the age-dependent formation of amyloid plaques, we stained brain sections of murine apoE-deficient hAPP/apoE3 mice, hAPP/apoE4 mice, and hAPP mice with thio-S. No thio-S-positive plaques were detected at 6–7 and 12–15 months of age in any of these mice (Figs. 4, 5). By 19–24 months of age, numerous plaques were found in the hippocampus of hAPP/apoE4 mice (Figs. 4, 5), whereas only few plaques were detected in the hippocampus of age-matched hAPP/apoE3 mice (Figs. 4, 5) or hAPP mice lacking apoE (data not shown). Plaques were most numerous in the stratum lacunosum in hAPP/apoE4 mice and almost completely restricted to this region in hAPP/apoE3 mice (Fig. 4). Thio-S-positive plaques were detected in the neocortex in one of seven hAPP/apoE4 mice and none of eight hAPP/apoE3 mice (data not shown).

To detect amyloid deposits that might be too diffuse to stain with thio-S, we stained brain sections of hAPP/apoE3 and hAPP/apoE4 mice with a polyclonal anti-Aβ antibody (R1282). Like thio-S-positive plaques, Aβ-IR deposits were detected only at 19–24 months of age but not at 6–7 or 12–15 months of age (Fig. 4). Aβ-IR deposits were detected primarily in the strata radiatum, oriens, and lacunosum of the hippocampus and were more numerous and dense in hAPP/apoE4 mice than in hAPP/apoE3 mice (Fig. 4). Only two of seven hAPP/apoE4 and none of eight hAPP/apoE3 had Aβ-IR deposits in the neocortex (data not shown).

Notably, at 12–15 months of age, hAPP/apoE4 mice already had a significant loss of SYN-IR presynaptic terminals and ChAT-IR fibers in the stratum lacunosum, although they had not yet formed plaques or diffuse amyloid deposits (Figs. 2–5). In 19- to 24-month-old hAPP/apoE4 mice, plaque load did not correlate with levels of SYN-IR presynaptic terminals or ChAT-IR fibers (Spearman's rank correlation). Furthermore, hAPP/apoE3 and hAPP/apoE4 mice showed marked differences in plaque load in the stratum lacunosum at 19–24 months of age but very similar losses of SYN-IR presynaptic terminals and ChAT-IR fibers (Table 1, Figs. 2–5). hAPP/apoE3 and hAPP/apoE4 mice showed an age-dependent decline in SYN-IR presynaptic terminals and ChAT-IR fibers in the neocortex (Figs. 2, 3), although Aβ-IR deposits were detected in this region in only two hAPP/apoE4 mice and thio-S-positive plaques were detected in only one hAPP/apoE4 mouse (data not shown). Lastly, no Aβ-IR deposits or plaques were found in the medial septum and nucleus basalis magnocellularis (data not shown), which give rise to the ChAT-IR fibers of the neocortex and hippocampus, respectively.

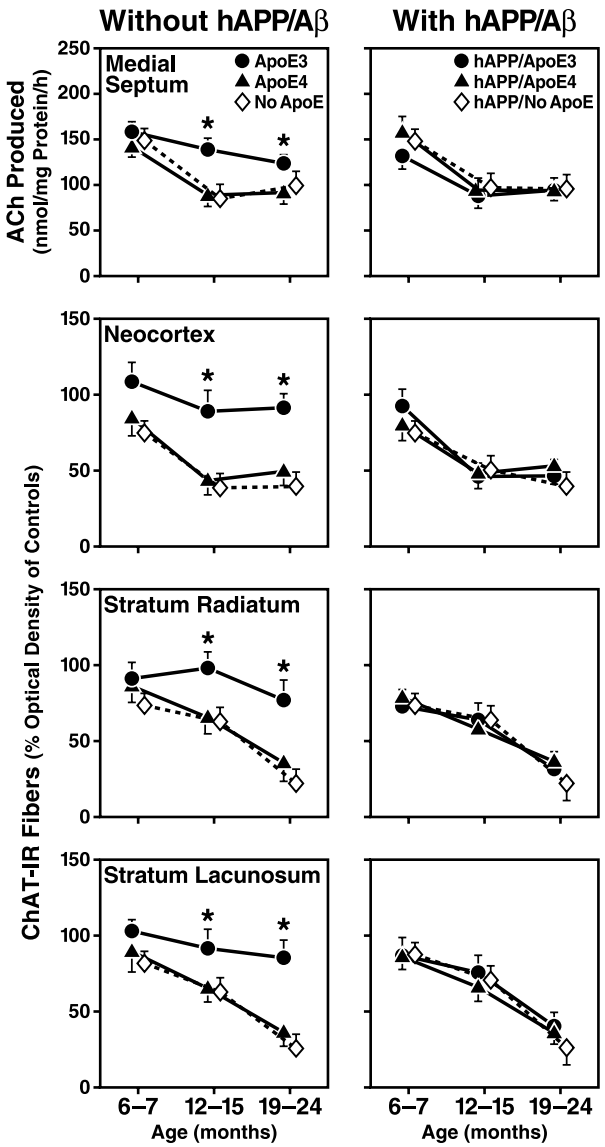

Figure 3. Age-related changes in ChAT activity (*top row*) and ChAT-IR fibers (*bottom rows*) in apoE singly transgenic (*left*) and hAPP/apoE doubly transgenic (*right*) mice lacking murine apoE. Littermates expressing neither human nor murine apoE served as additional controls. ChAT activity was measured in the medial septum. Levels of ChAT-IR fibers in the strata radiatum and lacunosum of the hippocampus and in the neocortex were expressed as percentage of corresponding levels in nontransgenic, wild-type (*Apoe*$^{+/+}$) control mice. Compared with apoE3 mice, apoE4 mice showed a loss of ChAT activity and ChAT-IR fibers at 12–15 and 19–24 months of age but not at 6–7 months of age. hAPP/apoE mice showed an age-related loss of ChAT regardless of whether they expressed apoE3 or apoE4. Age-related losses of ChAT in apoE-deficient mice with or without hAPP expression were similar to those in apoE4 mice. Results represent means ± SEM; n = 3–10 (ChAT activity) and n = 4–7 (ChAT-IR fibers) mice per genotype and age range; $^{*}p < 0.05$, age-matched mice expressing apoE3 versus apoE4 (Tukey–Kramer test). ChAT activity measurements for one 19-month-old apoE3 mouse and one 12-month-old apoE4 mouse were excluded from the statistical analysis because they were well outside of the range of the other values.

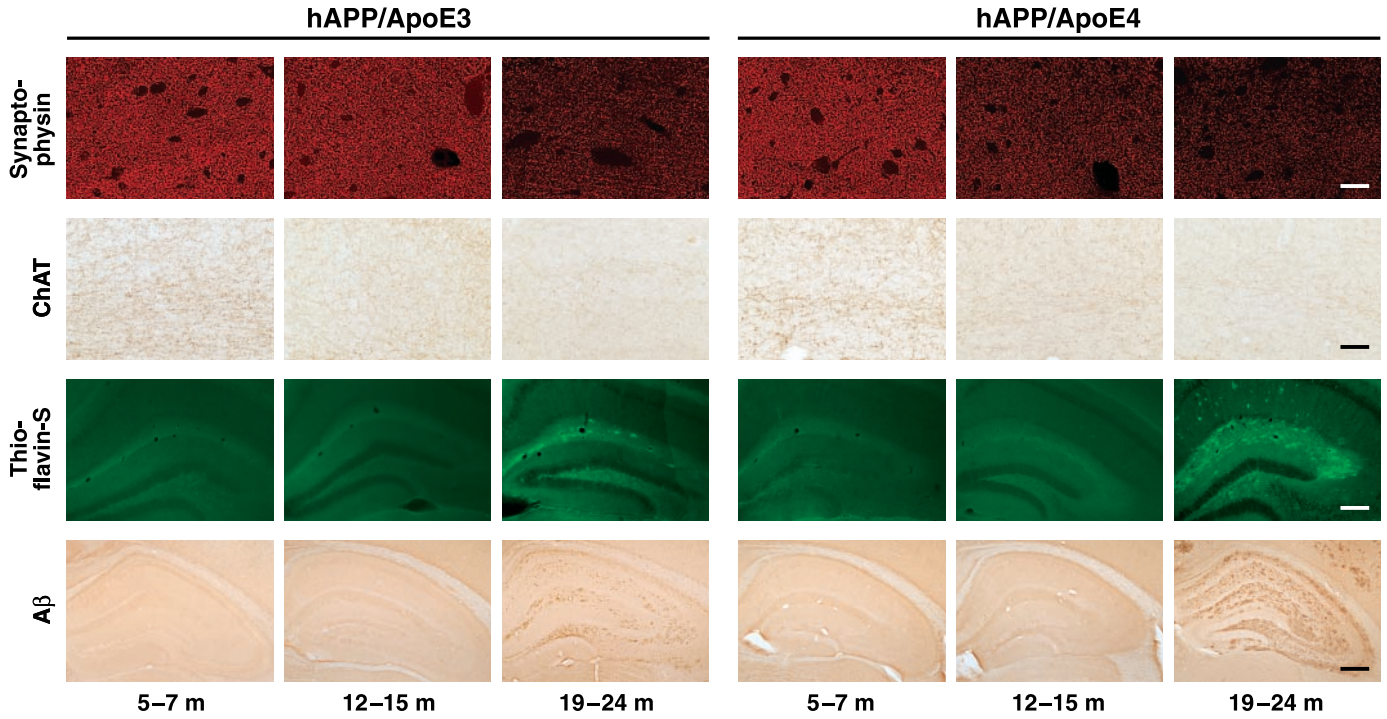

Figure 4. Age-dependent progression of neuropathological alterations in hAPP/apoE3 and hAPP/apoE4 mice. SYN-IR presynaptic terminals (stratum lacunosum) and ChAT-IR fibers (strata lacunosum and radiatum) of defined signal intensity were measured in the hippocampus. Age-dependent decreases in SYN-IR presynaptic terminals developed later in hAPP/apoE3 mice than in hAPP/apoE4 mice. Thio-S-positive plaques and Aβ-IR deposits in the hippocampus were detected only in the oldest age group, with more deposits found in hAPP/apoE4 mice than in hAPP/apoE3 mice. Note that 12- to 15-month-old hAPP/apoE4 mice had decreased levels of SYN-IR presynaptic terminals and ChAT-IR fibers but no amyloid deposits, and that 19- to 24-month-old hAPP/apoE4 mice had a larger amyloid burden than hAPP/apoE3 mice (Fig. 5), although at this age both groups had comparable decreases in SYN-IR presynaptic terminals and ChAT-IR fibers (Figs. 2, 3). Scale bars: *top row*, 30 μm; *second row*, 65 μm; *third row*, 250 μm; *fourth row*, 400 μm.

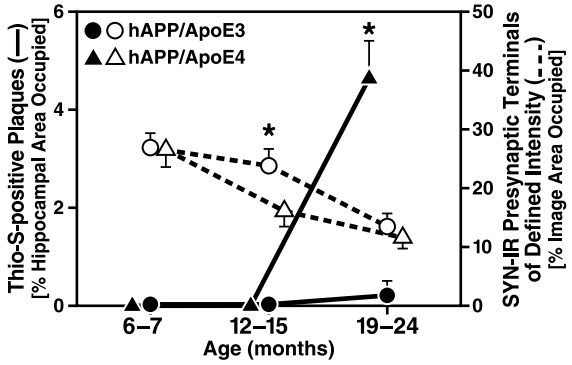

Figure 5. Age-dependent formation of thio-S-positive plaques in the hippocampus of hAPP/apoE mice. Brain sections were stained with thio-S and imaged by confocal microscopy (FITC filter setting). The hippocampal area occupied by thio-S-positive plaques was determined (*y*-axis on *left*, *solid symbols* and *lines*). Thio-S-positive amyloid plaques were detected only in the 19–24 month age group. hAPP/apoE4 mice had a significantly higher plaque load than hAPP/apoE3 mice. Results represent means ± SEM; $n = 4–7$ mice per genotype and age range; $^*p < 0.05$ by Mann–Whitney *U* test. Synaptophysin data (stratum lacunosum) from Figure 2 were superimposed (*y*-axis on *right*, *open symbols* and *dashed lines*) to highlight that the differential effects of apoE isoforms on plaques and SYN-IR presynaptic terminals occur at different ages.

DISCUSSION

The current study demonstrates that brain aging in the context of high levels of nondeposited human Aβ is associated with a progressive loss of SYN-IR presynaptic terminals, ChAT activity, and ChAT-IR fibers. The pace of the synaptic but not of the cholinergic decline was critically influenced by which isoform of human apoE was expressed in the brain. ApoE3 delayed the synaptic decline until old age, whereas apoE4 had no significant protective effects on synapses at any of the ages analyzed. Because synaptic deficits correlate well with the development of cognitive deficits in AD (DeKosky and Scheff, 1990; Terry et al., 1991; Langlais et al., 1993; Samuel et al., 1997; Sze et al., 1997; Brown et al., 1998), the differential effects of apoE3 and apoE4 identified here might relate closely to the effects of these isoforms on AD risk (E4 > E3) and age of onset (E4 < E3) (Corder et al., 1993; Farrer et al., 1997).

Studies of AD brains have identified a higher plaque burden in people with apoE4 than in people with apoE3 (Berr et al., 1994; Heinonen et al., 1995; Hyman et al., 1995; Ishii et al., 1997; Johnson et al., 1998; McNamara et al., 1998), and these observations have been confirmed in transgenic models expressing these human apoE isoforms in astrocytes (Holtzman et al., 2000a,b) or neurons (this study). These associations have widely been interpreted as evidence that apoE4 increases AD risk through its effect on amyloid deposition (Holtzman et al., 2000a,b; Selkoe, 2001). However, there are several reasons to consider alternative possibilities.

ApoE4 accelerates AD onset and worsens age-related neuronal decline during the early stages of aging but appears to have relatively little impact on the severity and progression of neuronal deficits in old age and during later stages of AD (Farrer et al., 1997; Bookheimer et al., 2000; Greenwood et al., 2000; Small et al., 2000; Yaffe et al., 2000; Caselli et al., 2001; Chapman et al., 2001; Reiman et al., 2001). In contrast, the effect of apoE4 on amyloid deposition is most prominent in advanced age, both in humans (Berr et al., 1994; Heinonen et al., 1995; Hyman et al., 1995; Ishii et al., 1997; Johnson et al., 1998; McNamara et al., 1998) and in transgenic mice (Holtzman et al., 2000a,b) (this study). ApoE4 increases the plaque burden not only in people with AD but also in nondemented individuals (Berr et al., 1994), underlining the potential dissociation between its effects on amyloid deposition and AD risk/onset. ApoE3 and apoE4 have differential effects on Aβ-dependent memory deficits in hAPP/apoE transgenic mice well before these mice develop amyloid deposits (Raber et al., 2000). Studies in human AD cases and in hAPP transgenic mice have demonstrated that AD-related synaptic and functional neuronal deficits correlate better with levels of nondeposited Aβ than with plaque load (Holcomb et al., 1999; Hsia et al., 1999; Lue et al., 1999; McLean et al., 1999; Mucke et al., 2000; Raber et al., 2000). In addition, several recent studies of people with one or two *APOE* ε4 alleles but without overt AD revealed decreased brain activity, decreased visual attention, and subtle memory impairments in ε4 carriers compared with people with other *APOE* alleles (Caselli et al., 1999; Bookheimer et al., 2000; Greenwood et al., 2000; Small et al., 2000; Reiman et al., 2001). These findings lend support to the notion that the critical effects of apoE isoforms on AD risk precede the clinical manifestation of AD by many years and, hence, are most likely independent of the prominent plaque formation that occurs during the later stages of the illness.

In the current study, we also detected a higher plaque load in old hAPP/apoE4 mice than in old hAPP/apoE3 mice. However, several findings suggest that this difference does not account for the differential effects of apoE isoforms on age- and Aβ-dependent synaptic and cholinergic decline. First, loss of SYN-IR presynaptic terminals and ChAT-IR fibers in hAPP/apoE4 mice clearly preceded plaque formation. Second, old hAPP/apoE3 and hAPP/apoE4 mice had comparable synaptic and cholinergic deficits but markedly different plaque loads. Third, the age-dependent loss of SYN-IR presynaptic terminals and ChAT-IR fibers also affected the neocortex, which was mostly devoid of plaques and Aβ deposits. Thus, it is likely that a plaque-independent mechanism accounts for the differential neuroprotective effects observed in our study.

At 12–15 months of age, neocortical levels of SYN-IR presynaptic terminals were significantly lower in hAPP/apoE4 mice than in hAPP/apoE3 mice, although these groups had similar levels of human Aβ1–x, Aβ1–42, and apoE in the neocortex. Thus, the difference in synaptic integrity in hAPP/apoE3 and hAPP/apoE4 mice was not caused by differences in the abundance of these transgene products. Neuroprotective activities of apoE3 that might allow it to delay age- and Aβ-dependent synaptic deficits include promotion of neurite extension (Nathan et al., 1994; Holtzman and Fagan, 1998) and synapse formation (Mauch et al., 2001), stabilization of microtubules (Strittmatter et al., 1994; Nathan et al., 1995) and endosomal–lysosomal membranes (Ji et al., 2002), and protection against oxidative stress (Miyata and Smith, 1996). Additional studies are needed to determine which of these mechanisms is most important in relation to age- and Aβ-dependent neuronal deficits *in vivo*.

Like the neuronal deficits identified in the current study, behavioral deficits in hAPP/apoE4 mice were seen in both males and females (Raber et al., 2000). In contrast, behavioral deficits in singly transgenic mice expressing apoE4 in the absence of hAPP/Aβ were seen only in females (Raber et al., 1998, 2000). Recent findings suggest that endogenous androgens protect singly transgenic male mice against apoE4-dependent behavioral deficits, and that this protection is relative rather than absolute (Raber et al., 2002).

Although apoE4 failed to prevent the age-dependent synaptic and cholinergic decline that occurs in murine apoE-deficient mice (with or without Aβ expression), it did not worsen it. This finding may be consistent with a loss of neuroprotective functions of apoE4 compared with apoE3. However, it could also reflect the gain of an adverse activity that counteracts or interferes with beneficial activities of the holoprotein (Raber et al., 1998, 2000; Tolar et al., 1999; Buttini et al., 2000; Huang et al., 2001). The $112_{Cys \rightarrow Arg}$ substitution, which differentiates apoE4 from apoE3, affects many aspects of apoE, including its conformation, stability, and binding to lipids and other molecules (Dong and Weisgraber, 1996; Ji et al., 1998; Huang et al., 2001; Raffaï et al., 2001). Determining whether these or other factors account for the lack of neuroprotective effects of apoE4 and its role in AD is an important objective.

Interestingly, overexpression of human Aβ failed to further augment the age-related synaptic and cholinergic deficits in mice lacking apoE. This finding may be consistent with the notion that the formation of neurotoxic Aβ species depends on the association of Aβ with "pathological molecular chaperones," such as apoE and apoJ (Wisniewski and Frangione, 1992; Oda et al., 1995; Ma et al., 1996; Permanne et al., 1997). In the presence of murine apoE (Hsia et al., 1999; Mucke et al., 2000; Masliah et al., 2001) or human apoE (this study), Aβ clearly did affect the integrity or function of presynaptic terminals and cholinergic neurons. Here, Aβ interfered with the ability of apoE3 to prevent loss of SYN-IR presynaptic terminals in the oldest group of mice and to prevent loss of ChAT-IR fibers at all ages analyzed.

It is interesting to speculate how these results might relate to findings obtained in epidemiological and clinicopathological studies in humans. As in our mouse models, the differential effects of apoE3 and apoE4 on the development of AD manifestations were apparent primarily during early but not late stages of the aging process (Farrer et al., 1997; Bookheimer et al., 2000; Greenwood et al., 2000; Small et al., 2000; Yaffe et al., 2000; Chapman et al., 2001; Reiman et al., 2001). The therapeutic effect of acetylcholinesterase inhibitors during early stages of AD suggests an early impairment of cholinergic functions (Cummings, 2000; Nakano et al., 2001), and there is evidence that the responsiveness to these compounds is influenced by apoE genotype (Farlow et al., 1998). However, biochemical and neuropathological alterations of the cholinergic system have been detected primarily in later stages of the disease, and their modification by apoE isoforms remains controversial (Poirier et al., 1995; Soininen et al., 1995; Allen et al., 1997; Arendt et al., 1997; Salehi et al., 1998; Gilmor et al., 1999; Bronfman et al., 2000; Corey-Bloom et al., 2000; Tiraboschi et al., 2000; Sjögren et al., 2001). Some of the reported discrepancies probably reflect genuine differences in the brain regions, AD populations, or disease models analyzed, but technical differences may also play a role (see Materials and Methods).

In conclusion, apoE3 and apoE4 have differential effects not

only on amyloid deposition and plaque-associated neuritic dystrophy but also on AD pathologies that appear to be primarily plaque independent, particularly loss of SYN-IR presynaptic terminals. Differences in the ability of these apoE isoforms to protect the brain against nondeposited toxic Aβ species could contribute to their effects on AD risk and onset. Drugs that simulate apoE3 activities or convert apoE4 into a molecule with apoE3-like function might delay both plaque-dependent and plaque-independent neuronal deficits in the many *APOE* ϵ4 carriers afflicted with or at risk for AD.

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