Expression of Human Apolipoprotein E4 in Neurons Causes Hyperphosphorylation of Protein Tau in the Brains of Transgenic Mice

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Epidemiological studies have established that the epsilon 4 allele of the *ApoE* gene (*ApoE4*) constitutes an important risk factor for Alzheimer's disease and might influence the outcome of central nervous system injury. The mechanism by which *ApoE4* contributes to the development of neurodegeneration remains unknown. To test one hypothesis or mode of action of *ApoE*, we generated transgenic mice that overexpressed human *ApoE4* in different cell types in the brain, using four distinct gene promoter constructs. Many transgenic mice expressing *ApoE4* in neurons developed motor problems accompanied by muscle wasting, loss of body weight, and premature death. Overexpression of human *ApoE4* in neurons resulted in hyperphosphorylation of the microtubule-associated protein tau. In three independent transgenic lines from two different promoter constructs, increased phosphorylation of protein tau was correlated with *ApoE4* expression levels. Hyperphosphorylation of protein tau increased with age. In the hippocampus, astrogliosis and ubiquitin-positive inclusions were demonstrated. These findings demonstrate that expression of *ApoE* in neurons results in hyperphosphorylation of protein tau and suggests a role for *ApoE* in neuronal cytoskeletal stability and metabolism. (*Am J Pathol* 2000, 156:951-964)

The epsilon 4 allele of the apolipoprotein E gene on chromosome 19q13.2 (*ApoE4*) is associated with late-onset and sporadic Alzheimer's disease (AD), as confirmed by many groups since the initial reports.^{1,2} *ApoE4* causes earlier onset of the disease in an allele-dose-dependent manner and increases the risk for its carrier by 1 order of magnitude. In addition, the ϵ 4 allele of apolipoprotein E has been associated with increased plaque load³⁻⁵ and with early onset of AD-related neurofibrillary changes in young individuals.⁶ Recently, genetic studies reported polymorphisms in the promoter region of the human *ApoE* gene associated with AD.⁷⁻¹⁰ These

might pertain to the observation that, in the brains of AD patients, *ApoE* messenger RNA (mRNA) was increased.¹¹ In addition to its well-documented role in AD, the *ApoE4* allele has been implicated in poorer neurological recovery to head injury, cerebral hemorrhage, and cognitive status after cardiac bypass surgery.¹²⁻¹⁷ These experiments provide epidemiological evidence for the close relationship between *ApoE* and AD or between *ApoE* and the outcome in nervous system injury, but do not provide an explanation for its mechanism of action within the nervous system.

The demonstration that the mainly astrocytic protein *ApoE* can also be found in human neurons might be important in assessing its role within the nervous system. Immunocytochemically, *ApoE* was demonstrated in human hippocampal neurons of AD patients and aged individuals¹⁸ and in cortical neurons of elderly.¹⁹ Recently, human brain neurons have been shown to synthesize *ApoE*. *In situ* hybridization revealed *ApoE* mRNA in the neurons of the CA1 to CA4 region of the hippocampus, the granule cell layer of the dentate gyrus, and many neurons in the frontal cortex.²⁰ In addition, transgenic mice that overexpress large fragments of the human *ApoE* locus, including the human *ApoE* promoter, also showed neuronal expression of human *ApoE*.²¹⁻²³ It is interesting that the regions where neuronal *ApoE* was present are most vulnerable in developing neurofibrillary tangles.^{20,24} Also, human neuroblastoma cells were shown to synthesize *ApoE* mRNA.^{25,26} In rodent brain, *ApoE* has been demonstrated in central neurons only after experimental brain injury.²⁷⁻³⁰ Numerous cell culture experiments have demonstrated receptor-mediated uptake of *ApoE* in neurons, and large effects on neuritic outgrowth and morphology were found.³¹⁻³⁷ *In vitro*, protein-protein interactions of *ApoE* with intracellular microtubule-as-

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Figure 1. Generation and characterization of different *ApoE4* transgenic mouse strains. **A:** Schematic representation of minigene constructs synthesized as described in the text. On the lower lines are indicated the *StuI* restriction site and size of restriction fragments used in genotyping by Southern blotting. The polymerase chain reaction primers (P78 and P79) used for routine genotyping are also indicated. **B:** Northern blot analysis of representative mice from the seven different *ApoE4* transgenic strains. Total RNA (10 μ g) was sequentially analyzed for expression of human ApoE mRNA (human-specific probe) and for actin mRNA. RNA from an ApoE knockout mouse and a wild-type mouse (wt) is included. **C:** Western blot of proteins extracted from the brains of the same mice as used in panel **B**, except for the ApoE knockout mice. A human-specific anti-human ApoE antibody was used (see Materials and Methods).

sociated proteins (MAP2c, tau) were observed.^{2,38} These results support an intracellular role for ApoE in neuronal pathology in AD and suggest that ApoE might be more directly involved in disruption of the neuronal cytoskeleton.

To study the repercussions of ApoE expression in different cell types in the brain, we have generated 25 independent founder transgenic mice that overexpress human ApoE4 in neurons and/or astrocytes. We have used four different gene promoter constructs derived from either the mouse *Thy1* gene,³⁹ the human *GFAP* gene,⁴⁰ the human *PDGF- β* gene,⁴¹ or the mouse *PGK* gene.⁴² The initial phenotypic characterization demonstrated strong neuronal expression in the hippocampus and cortex of Thy1-ApoE4 and PDGF-ApoE4 transgenic mice, whereas the PGK-ApoE4 transgenic mice showed neuronal expression over the entire brain. As expected, in the GFAP-ApoE4 transgenic mice, expression was restricted to astroglia. Surprisingly, with a panel of protein tau-specific antibodies, hyperphosphorylation of the microtubule-associated protein tau became evident in the transgenic mice that overexpressed human ApoE4 in neurons. This might provide a clue to how ApoE could influence or disturb neuronal cytoskeletal stability.

Materials and Methods

Constructs and Generation of ApoE4 Transgenic Mice

All constructs were based on a 5.5-kb *Bam*HI-*Hind*III fragment of the human *ApoE4* gene (Figure 1). The mouse *thy1* gene, cloned as an 8.1-kb *Eco*RI genomic fragment,³⁹ was adapted by replacing a 1.5-kb *Bam*HI-*Xho*I fragment with a synthetic *Xho*I oligonucleotide adapter, in which the human ApoE4 *Bam*HI-*Hind*III fragment was ligated. The *PDGF* gene promoter^{41,43} and the *GFAP* gene promoter^{40,44} were combined with the 1.5-kb *Eco*RI-*Xho*I fragment containing the mouse *thy1* gene 3'UTR with polyadenylation signal. For the *PGK-ApoE4* construct, the 5.5-kb human ApoE4 fragment was ligated in the *Pst*I site of a PGK expression cassette.^{42,45}

Minigenes were partially sequenced. Thy1-ApoE4, PDGF-ApoE4, and GFAP-ApoE4 vectors were linearized with *Pvu*II, and the PGK-ApoE4 vector was linearized with *Eco*RV and *Xho*I. Subsequently, purified constructs were diluted in 10 mmol/L Tris, 0.2 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.4, to a concentration of 3 μ g/ml for microinjection into 0.5-day pre-nuclear embryos isolated from superovulated FVB/N female mice. The in-

jected embryos were either cultured overnight to reach two-cell stage or were immediately transferred to the oviduct of pseudopregnant CD1 foster mice, with similar results. Offspring was weaned and tail biopsies were taken for DNA isolation and Southern blotting. Genomic DNA (10 μ g) was digested with *Stu*I, separated by gel-electrophoresis, transferred, and hybridized by standard procedures with a 672-bp probe generated by polymerase chain reaction from the human *ApoE4* gene.

Routinely, transgenic offspring was identified by polymerase chain reaction, using primers in exon 4 of the human *ApoE4* gene as follows: forward primer, 5'-GCGGGCACGGCTGTCCAAG (P78); reverse primer, 5'-GGGGTGGCGTGGGGTGCAT (P79). Mouse tail biopsies were digested overnight at 55°C with 1 mg/ μ L proteinase K in 50 mmol/L Tris-HCl (pH 8.5), 1 mmol/L EDTA, 0.5% sodium dodecyl sulfate, 0.1 mol/L NaCl. The enzyme was inactivated by boiling for 10 minutes, and diluted samples were analyzed by polymerase chain reaction with the following program: 2 minutes at 95°C, followed by 30 cycles of 1 minute at 95°C, 1 minute at 60°C, 1 minute at 72°C, and a final extension of 15 minutes at 72°C.

Histology and Immunohistochemistry

Anesthetized mice were perfused with phosphate-buffered saline followed by 4% paraformaldehyde. Whole brain was fixed for 16 hours in 4% paraformaldehyde at 4°C and washed in phosphate-buffered saline, dehydrated, and embedded in paraffin for sectioning (5–7 μ m). Alternatively, vibratome sections of 40 μ m were cut and transferred to microtiter wells in phosphate-buffered saline containing 0.5% sodium azide at 4°C.

Paraffin sections were routinely stained with hematoxylin-eosin (H&E), cresylviolet (Nissl stain), and Garvey silver stain by standard procedures. Immunohistochemical staining of sections was performed by standard procedures with commercially available antibodies: ApoE (Dako, Glostrup, Denmark), GFAP (Dako), ubiquitin (Dako), AT8 (Innogenetics, Ghent, Belgium), PHF1 and Alz50 (P. Davies), and B19 (J. P. Brion). Vibratome sections were treated with 0.6% (w/v) hydrogen peroxide to quench the endogenous peroxidase activity. After rinsing, sections were incubated in Tris-buffered saline, containing 10% goat serum and 0.2% Triton-X-100 for 2 hours, before incubation with antibodies against ApoE (1:10,000, overnight at room temperature). For immunohistochemical staining with B19 (1:500), the detergent was omitted. Secondary antibody was biotinylated goat-anti-rabbit immunoglobulin G (IgG) followed by StreptAB-Complex/HRP (Dako). Final staining was developed with 0.075% 3,3-diaminobenzidine and 0.01% (w/v) hydrogen peroxide. Staining with the monoclonal antibodies AT8 (1:80), PHF1 (1:500), and Alz50 (1:50) was performed with the Dako ARK staining method, by the instructions of the manufacturer. Sections were mounted on gelatin-coated glass slides, counterstained with hematoxylin, dehydrated, and mounted.

In Situ Hybridization

The sense and antisense human ApoE4 probes were synthesized from a pBluescript (SK-) vector (Stratagene, La Jolla, CA) in which we cloned a 161-bp *Eco*NI/*Eco*47III restriction fragment from the human *ApoE4* gene. The plasmid was linearized with either *Eco*RI or *Cla*I and transcribed with T7 and T3 RNA polymerase, respectively, in the presence of [³⁵S]UTP. Paraffin sections (6 μ m) were transferred on silanated glass slides, dewaxed, and rehydrated through an ethanol series. Sections were digested with proteinase K (20 μ g/ml), postfixed in 4% paraformaldehyde, and treated with 0.25% acetic anhydride in 0.1 mol/L triethanolamine-HCl. Sections were hybridized overnight in 50% deionized formamide, 0.3 mol/L NaCl, 20 mmol/L Tris-HCl, 5 mmol/L EDTA (pH 8.0) with 10% dextran sulfate, 1× Denhardt's solution, 0.5 mg/ml yeast RNA, and 10 mmol/L dithiothreitol and supplemented with the appropriate radiolabeled riboprobe. After stringency washes and ribonucleaseA treatment, sections were dehydrated, and dipped in photographic emulsion (LM-1, Amersham) and exposed for 1 week.

Analysis of RNA and Protein

Brains of 6-week-old transgenic offspring were removed from the skulls as quickly as possible; for each, one hemisphere was used for analysis of ApoE mRNA, and the other was used for analysis of ApoE protein expression. Analysis of RNA and protein was done as previously described.³⁹ ApoE protein was detected with a polyclonal antibody to human ApoE4 (Dako).

In brains from 3- to 24-month-old transgenic offspring, one hemisphere was fixed in 4% paraformaldehyde and processed for immunohistochemistry, and the other was used for Western blot analysis of protein tau. Samples were homogenized in 10 vol of buffer consisting of 0.1 mol/L 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.4, 0.5 mmol/L MgCl₂, 0.1 mmol/L EDTA, 1 mmol/L ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid. Just before use, the following proteinase and phosphatase inhibitors were added: 1 mmol/L dithiothreitol, 5 μ g/ml leupeptin, 5 μ g/ml pepstatin, 200 μ mol/L phenylmethylsulfonyl fluoride, 20 mmol/L NaF, 100 mmol/L Na₃VO₄, 1 μ mol/L okadaic acid, 1 mmol/L EDTA, 5 μ g/ml soybean trypsin inhibitor, 1% sodium deoxycholate, 1% Triton-X-100, and 0.1% sodium dodecyl sulfate (final concentrations). The homogenized suspension was centrifuged for 30 minutes at 100,000 × *g* at 4°C. Supernatants were stored at –70°C until use. Protein concentration was measured with the BioRad Dc protein Assay (BioRad, Hercules, CA). Before loading on the gel, 2% sodium chloride and 5% β -mercaptoethanol were added. Samples were heated at 100°C for 10 minutes in tightly capped tubes, chilled on ice for 30 minutes, and centrifuged for 15 minutes at 12,000 × *g* at 4°C. To the supernatants, 2% sodium dodecyl sulfate, 1% β -mercaptoethanol, and 10% glycerol blue (final concentrations) were added. After heating at 95°C for 10 minutes, proteins were separated by denaturing sodium dodecyl sul-

Table 1. Expression Patterns of Promoters According to the Literature

Promotor	Expression in	Time of expression		References
		Embryonal	Postnatal	
Thy1	Neurons	—	p15, onset; p22, full expression	46
	Hippocampus			47
	Cortex			50
				49
GFAP	Astroglia	From E12.5–E13.5	+	40
PDGF	Neurons	E9.5–E10.5, periaortic mesenchym; E12.5–E16.5, mesenchym	+	44
	Hippocampus			43
	Cortex			41
	Hypothalamus			
PGK	Cerebellum	E3.5–E4.5, blastocyst	Variable expression	
	Also in other tissues			51
	All tissues in brain			52
	Large neurons			
	Cortex			
	Hippocampus			
	Dentate gyrus			

fate-polyacrylamide gels (NOVEX, Frankfurt-au-Main, Germany). Western blotting was performed with monoclonal antibodies PHF1, AT8, AT180, and TAU5. The monoclonal antibodies AT8 and AT180 (Innogenetics) recognize protein tau phosphorylated at serine-202 and threonine-205 (AT8), and threonine-231 (AT180). Monoclonal antibody PHF-1 (kindly provided by P. Davies) is specific for protein tau phosphorylated at serine-396 and serine-404. Total tau protein was detected with monoclonal antibody TAU5 (Pharmingen, San Diego, CA), which recognizes a phosphorylation-independent epitope of tau protein. Dephosphorylation of protein tau was performed with 300 U/ml alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany), incubated at 37°C for 1 h.

Quantitative analysis of Western blots, assessed by densitometric scanning, was normalized, and 3 to 4 mice per transgenic strain and per age group were analyzed.

Results

Generation of Transgenic Mice Expressing Human ApoE4 with Four Different Gene Promoters

Transgenic mice were generated that overexpress human ApoE4 under the control of well-characterized promoters, derived from the following four genes: the mouse *Thy1* gene, the human *PDGF* gene, the human *GFAP* gene, and the mouse *PGK* gene. The constructs are schematically represented in Figure 1A, and the known expression patterns of the gene promoters are summarized in Table 1.

The linearized constructs were injected into mouse pre-nuclear embryos isolated from superovulated FVB female mice. Fifteen independent Thy1-ApoE4, five PDGF-ApoE4, five GFAP-ApoE4, and two PGK-ApoE4 founders were obtained. For each construct, two independent transgenic founders with high expression of ApoE4 and with good breeding performance were selected, except

for PGK-ApoE4, for which only one strain was retained. The following notations are used to designate individual lines of the different constructs: tae-II (Thy1-ApoE4 line 2), tae-XIII (Thy1-ApoE4 line 13), pae-II (PDGF-ApoE4 line 2), pae-V (PDGF-ApoE4 line 5), pgk-I (PGK-ApoE4 line 1), gae-I (GFAP-ApoE4 line 1), and gae-III (GFAP-ApoE4 line 3). Wt is used to designate wild-type mice and ApoE^{−/−} to designate ApoE knockout mice.

Analysis of Expression of Human ApoE4

Expression of human ApoE4 in brain was demonstrated and measured by Northern and Western blotting in F1 offspring (2 months old), from all founders. Comparative analysis of expression of human ApoE4 mRNA and protein in brain from the selected transgenic mouse strains is shown in Figure 1, B and C, respectively. Northern blotting demonstrated expression in all tissues examined in the PGK-ApoE4 transgenic mice, whereas *in situ* hybridization showed expression already in postimplantation embryos at E5.5.

Quantitation of relative levels of ApoE4 mRNA by densitometric scanning, showed that expression levels in the tae-II and tae-XIII transgenic mice were, respectively, 3- and 4.4-fold higher than in pae-II transgenic mice. In the gae-I transgenic mice, ApoE4 expression levels were 4.5-fold higher than in pae-II transgenic mice.

The spatial distribution of human ApoE4 mRNA was visualized by *in situ* hybridization in brain of adult transgenic mice. Expression patterns resulting from the different gene promoters were as expected.^{40,41,43,44,46–52} Strong hybridization signals in pyramidal cells of the hippocampus, in the granular cells of the dentate gyrus, and in the deeper layers of the cerebellar cortex were detected in the brains of Thy1-ApoE4 mice (Figure 2, A and C). Expression was further evident in neurons of the amygdala and striatum. Signals were most restricted to neuronal cell bodies, with minor hybridization signals in the proximal ends of some neurites. PDGF-ApoE4 transgenic mice strongly expressed in the neuronal cell bod-

Figure 2. *In situ* hybridization of brains of *ApoE4* transgenic and Wt mice. Sections were probed with the antisense human-specific *ApoE* probe and counterstained with toluidine blue. Coronal brain sections of Thy1-*ApoE4* (A), and GFAP-*ApoE4* (B) mice. Scale bar, 1 mm. Bar indicated in A also applies to B. C: Detail of the cortex of Thy1-*ApoE4* (C) and GFAP-*ApoE4* (D) mice. Parietal associative cortex of Wt (E), PDGF-*ApoE4* (F), and PGK-*ApoE4* (G) mice. Detail of the cortex of PDGF-*ApoE4* (H) and of the pyramidal cells of the CA1 region of the hippocampus of PGK-*ApoE4* (I) mice. Scale bar, 50 μ m. Bar indicated in G also applies to E and F. Bar indicated in I also applies to C, D, and H. Py, pyramidal cell layer of the hippocampus.

ies of the cortex, in pyramidal cells of the hippocampus, and in granular cells of the dentate gyrus, but only weakly in amygdala (Figure 2, F and H). The PGK-*ApoE4* mice showed diffuse expression in cells over the entire brain,

with pyramidal and granular cells of the hippocampus and cells in the choroid plexus displaying stronger signals (Figure 2, G and I). In contrast, but as expected, GFAP-*ApoE4* mice expressed the transgene strongly in

astrocytes (Figure 2, B and D). The presence of weak signals in layer II and V neurons in the cortex could not be excluded.

The distribution of the human ApoE4 protein in transgenic mouse brain as analyzed immunohistochemically on vibratome sections (Figure 3) was similar to the distribution of its mRNA in all mice analyzed from the seven transgenic strains. In the Thy1-ApoE4, PDGF-ApoE4, and PGK-ApoE4 transgenic mice, we observed somatodendritic staining of neurons (Figure 3, A, C, D, F, G, and J), whereas in the GFAP-ApoE4 transgenic mice, ApoE immunoreaction was restricted to astrocytes (Figure 3, H and I). In the Thy1-ApoE4 transgenic mice, a granular staining pattern, probably corresponding to vesicular structures, was present in some dendrites (Figure 3D).

Spontaneous Behavior of ApoE4 Transgenic Mice

All transgenic mice from all constructs appeared normal during the first 2 to 3 months of life. Thereafter, many mice progressively manifested motor problems accompanied by muscle wasting, loss of total body weight, and premature death. These signs were evident in the five transgenic strains with Thy1-ApoE4 (3 to 12 months old), PDGF-ApoE4, and PGK-ApoE4 constructs (12 months or older).

Thy1-ApoE4 transgenic mice with highest expression levels progressively developed the most severe phenotype. At around 6 months of age, about 60% of the mice in strain tae-II displayed bleeding excoriations on the eyelids, which appeared swollen. Close observation revealed these to result from scratching with the hind legs. Homozygous animals developed the phenotype at an earlier age than heterozygous animals of the same strain, housed in the same conditions. Muscle wasting and loss of bodyweight (30%) accompanied these symptoms, most likely due to the progressive inability to climb to the roof of the cage for feeding. This was further reflected in the increased mortality of the Thy1-ApoE4 transgenic mice: at 6 months, already 66% of transgenic mice in the highest expressing strain (tae-XIII) and 25% in the lower expressing Thy1-ApoE4 strain (tae-II) succumbed (Figure 4).

Similar but much less intense or severe phenotypic characteristics appeared in a limited number of transgenic PDGF-ApoE4, PGK-ApoE4, and GFAP-ApoE4 animals, when more than 12 months old. In these transgenic mouse strains, the mortality was only marginally higher than in nontransgenic mice of the same age and gender housed under identical conditions (Figure 4).

Hyperphosphorylation of Protein tau in Thy1-ApoE4 Transgenic Mice

We investigated protein tau phosphorylation in mice transgenic for human apolipoprotein E4 by Western blotting of brain homogenates. tau proteins are separated as a complex set of protein species reflecting both the ex-

pression of several isoforms and the presence of differentially phosphorylated species.⁵³

Protein tau phosphorylation was comparatively analyzed with four specific monoclonal antibodies, ie, AT8, AT180, PHF1, and TAU5. Western blot analysis of brain homogenates of transgenic mice showed that the microtubule-associated protein tau became hyperphosphorylated in mice expressing ApoE4 in neurons, which was demonstrated in two independent Thy1-ApoE4 transgenic lines. An increase in protein tau phosphorylation appeared in Thy1-ApoE4 line 13 (tae-XIII) transgenic mice of 3 months and was very prominent in mice of 18 months (Figure 5A). In line tae-II, with lower neuronal expression levels, increased protein tau phosphorylation appeared when mice were 7 months old, indicating that hyperphosphorylation of protein tau correlated with neuronal ApoE4 expression levels. The marked decrease in electrophoretic mobility of the immunoreactive protein tau isoforms, following immunoblotting with TAU5 antibody, confirmed the increase in phosphorylation of protein tau in the brain of Thy1-ApoE4 transgenic mice, demonstrated with antibodies AT8, AT180, and PHF1 (Figure 5A). Dephosphorylation of protein tau by pretreatment of brain extracts with alkaline phosphatase reduced or abolished the immunoreaction of the slower-migrating isoforms, detected with antibodies AT8 and TAU5 (Figure 7). Phosphorylation of protein tau was variable at all age groups, but was always higher in transgenic mice relative to age-matched Wt mice.

Protein tau phosphorylation was quantified by densitometric analysis of Western blots. In Thy1-ApoE4 line 2 (tae-II) transgenic mice, AT8 staining was 3.7-fold higher at 7 months and 12-fold higher at 14 months compared with age-matched Wt mice. PHF-1 staining was not significantly increased at 7 months, but was 4.1-fold higher at 14 months in tae-II transgenic mice relative to age-matched Wt mice (Figure 8). The 60-kD protein tau band, detected by the monoclonal antibody TAU5, was threefold more intensive in tae-II transgenic mice relative to age-matched Wt mice, when mice were 7 or 14 months old (Figure 8), whereas total amounts of protein tau were not different (Figure 8).

Hyperphosphorylation of protein tau became evident only in brain of PDGF-ApoE4 mice when 2 years old (Figure 6). Neither in GFAP-ApoE4 mice of two years old (Figure 6) nor in PGK-ApoE4 mice of 12 to 17 months old was an increase in protein tau phosphorylation ever observed.

Brain Histology

Histological and immunohistochemical analysis was performed on 28 transgenic and 11 Wt mice between 3 and 23 months old. The histological repercussion of ApoE4 overexpression was minimal as judged by H&E staining of brain sections of transgenic mice and age-matched Wt mice. The brain appeared microscopically normal and did not show signs of deviating architecture or neuronal loss.

Astrogliosis, assessed by immunostaining for GFAP, was evident in the brain of older ApoE4-transgenic mice

Figure 3. Immunohistochemistry for human ApoE4 in brains of transgenic and Wt mice. Sections were counterstained with hematoxylin. **A–C:** Cortical region of Thy1-ApoE4, Wt, and PDGF-ApoE4 mice, respectively. **D:** Detail of dendrites in cortex of a Thy1-ApoE4 mouse. **E–G:** Details of the pyramidal cells in the CA1 region of the hippocampus in Wt, PDGF-ApoE4, and Thy1-ApoE4 transgenic mice, respectively. **H** and **I:** Astrocytes in the entorhinal cortex of a GFAP-ApoE4 mouse. **J:** Astrocytes of neuronal cell bodies in the neocortex of a PGK-ApoE4 mouse. Scale bars, 50 μ m. Scale bar indicated in **A** also applies to **B**, **C**, and **H**. Scale bar indicated in **E** also applies to **G**, **F**, **I**, and **J**. Py, pyramidal cells of the hippocampus; cc, corpus callosum.

Figure 4. Survival of *ApoE4* transgenic mice: tae-XIII, *n* = 15; tae-II, *n* = 43; pae-II, *n* = 31; pae-V, *n* = 19; pgk-I, *n* = 14; gae-III, *n* = 21; wt, *n* = 30.

(Figure 9, A–F). Gliosis was most prominent in the neo-cortex, hippocampus, and amygdala of transgenic mice with highest neuronal expression levels, ie, in the Thy1-*ApoE4* and PDGF-*ApoE4* mice (Figure 9, A, B, and F). In

Figure 5. Comparison of protein tau hyperphosphorylation between Thy1-*ApoE4* (line tae-XIII) and wild-type (wt) mice at ages 3 to 18 months. Western blots shown are representative examples. Four independent tau-specific monoclonal antibodies (AT8, AT180, PHF1, and TAU5) were used. Goat-anti-mouse peroxidase was used as secondary antibody.

Figure 6. Comparison of protein tau hyperphosphorylation between 2-year-old PDGF-*ApoE4* (pae-II), GFAP-*ApoE4* (gae-I), and wild-type (wt) mice. Goat-anti-mouse peroxidase was used as secondary antibody.

the GFAP-*ApoE4* mouse, only minimal gliosis was present (Figure 9D).

Ubiquitin-containing inclusions were present in the neuropil of the stratum oriens of the hippocampus and in the fimbria hippocampi of Thy1-*ApoE4* and PDGF-*ApoE4* transgenic mice, but not in Wt mice (Figure 9, G–H). The inclusions were rounded or more angular in shape.

Figure 7. Dephosphorylated protein extract of a Thy1-*ApoE4* and wild-type mouse with alkaline phosphatase, compared with untreated (native) samples. AT8 and TAU5 monoclonal antibodies were used to detect bands.

Figure 8. Quantitative comparison of the levels of hyperphosphorylated protein tau immunoreactivities of 7- and 14-month-old Thy1-ApoE4 transgenic and age-matched control mice. Data are means $\pm$ SEMs (bars) of wild type ($\square$, $n = 3$) and Thy1-ApoE4 transgenic ($\blacksquare$, $n = 3$) mice. Data were obtained by densitometric analysis of immunoblots and normalized.

Immunohistochemical staining with B19, a polyclonal antiserum recognizing protein tau independent of its phosphorylation state, showed widespread axonal and somatodendritic staining in white matter fiber tracts, neocortex, hippocampus, and thalamus, which was not different in Thy1-ApoE4 transgenic mice relative to Wt mice (Figure 10A). This staining pattern was also similar to the one observed in rat brain.⁵⁴ Staining with the phosphate-dependent monoclonal antibody AT8 revealed reactivity mainly in the neocortex, hippocampus, and amygdala of Thy1-ApoE4 transgenic mice. Staining was most intense in old Thy1-ApoE4 transgenic mice (12–18 months), compared with age-matched Wt mice (Figure 10, B and C). The phosphorylation-dependent monoclonal antibody PHF1 stained neurons in the neocortex, thalamic nuclei, and the CA3 and CA4 regions of the hippocampus. Staining was again most intense in old Thy1-ApoE4 transgenic mice compared with age-matched Wt mice. Staining with

Figure 9. A–E: GFAP staining of the parietal associative cortex of Thy1-ApoE4 (A), PDGF-ApoE4 (B), PGK-ApoE4 (C), GFAP-ApoE4 (D), and Wt (E) mice. F: Detail of the hippocampus of a Thy1-ApoE4 mouse showing reactive astrogliosis. G and H: Ubiquitin positive inclusions (arrows) can be seen in the CA3 region of the hippocampus of Thy1-ApoE4 (G) and PDGF-ApoE4 (H) mice. Scale bar, 50 μ m. Scale bar indicated in A also applies to B, C, D, and E. Scale bar indicated in G also applies to H. Py, pyramidal cells of the hippocampus; CC, corpus callosum.

Figure 10. **A:** Staining with the polyclonal anti-tau antibody B19 shows a widespread somatodendritic localization in the parietal associative cortex of a Thy1-ApoE4 transgenic mouse 18 months old. **B:** Staining with the monoclonal phosphorylation-dependent antibody AT8 recording a slightly more intense somatodendritic staining in the parietal associative cortex of the Thy1-ApoE4 mice 18 months old, compared with aged-matched wild-type mice (**C**). Scale bar, 100 μ m.

the conformation-dependent monoclonal antibody Alz50 remained negative both in Wt and Thy1-ApoE4 transgenic mice. Neither immunohistochemistry nor silver staining revealed any neurofibrillary tangles or other types of neurofibrillary inclusions.

Immunohistochemistry for β A4 or silver staining did not reveal amyloid deposition in any of the ApoE4 transgenic mice.

Discussion

The impact of expression of human apolipoprotein E4 in different cell types in the brain was examined by generating transgenic mice expressing human ApoE4 in neurons or glial cells. Mice transgenic for human ApoE4 expressed the transgene exclusively in neurons when under control of the *Thy1* or *PDGF* gene promoters and in astrocytes when under control of the *GFAP* gene promoter, as expected. Remarkably, the expression in neurons resulted in hyperphosphorylation of protein tau, in addition to behavioral disturbances and morphological neuronal changes in the brain, such as ubiquitin-positive inclusions and astrogliosis.

In three independent transgenic mouse strains, neuronal expression of human ApoE4 resulted in protein tau hyperphosphorylation. The level of neuronal expression, in combination with aging, appeared to be the most important determining factors. Offspring of the highest expressing Thy1-ApoE4 transgenic line (line tae-XIII) displayed protein tau hyperphosphorylation when 3 months old, whereas mild overexpression, ie, in line pae-II, resulted in hyperphosphorylation only when mice were 2 years old. Within a particular age group of Thy1-ApoE4 transgenic mice, protein tau hyperphosphorylation was somewhat variable, as reflected by the levels of the immunoreactive isoforms detected in Western blotting with

specified monoclonal antibodies. Using the polyclonal phosphorylation-independent antibody B19, the somatodendritic localization of protein tau in cortical and hippocampal neurons was similar in old Thy1-ApoE4 transgenic and Wt mice. This result is in agreement with a previous study in rats, showing phosphorylated tau in the soma and dendrites of neurons in adult brain.⁵⁴ In addition, the brain of Thy1-ApoE4 transgenic mice, shown to exhibit increased protein tau phosphorylation on Western blotting, also showed increased immunoreactivity for phosphorylated tau with antibodies AT8 and PHF1 on brain sections.

The remarkable phenotypic parameter of all transgenic mice that express ApoE4 in neurons, ie, protein tau hyperphosphorylation, relates to *in vitro* studies illustrating isoform-specific interactions between ApoE and protein tau.² Our results demonstrate that, under specified conditions, ApoE4 can cause protein tau to become hyperphosphorylated directly, *in vivo*. Whether this is through a direct molecular interaction or indirectly, remains at present unclear.

Transgenic mice expressing ApoE4 in neurons under control of the neuron-specific enolase promoter were reported to have difficulties with learning in water maze tasks and with exploratory behavior at 6 months.⁵⁵ Humanized transgenic mice carrying genomic sequences for ApoE have been generated, showing neuronal expression of ApoE in addition to glial expression.²¹⁻²³ It would be interesting to know whether these mice would also show protein tau hyperphosphorylation at an older age. In the PDGF-ApoE4 transgenic mice, which have lower expression levels, tau hyperphosphorylation and motor impairments appeared only when mice were around 2 years old.

Other transgenic mice generated in our laboratory, expressing very high levels of unrelated proteins under

control of the same *Thy1* gene promoter, did not show protein tau hyperphosphorylation and remained completely normal throughout life. Therefore, we exclude the possibility of a general neurotoxic effect of overexpressing high levels of any protein in central neurons, under control of the *Thy1* gene promoter.

Protein tau hyperphosphorylation has been demonstrated in mice lacking ApoE,^{56,57} although conflicting results have been reported.⁵⁸ Because brain endogenous mouse ApoE levels remained unchanged in transgenic mice expressing human ApoE4 in neurons, the observed protein tau hyperphosphorylation cannot be attributed to a down-regulation or absence of endogenous mouse ApoE.

In apolipoprotein E transgenic mice generated with the same *GFAP* promoter as used here, ApoE proteins were secreted in high-density like lipoprotein particles.⁵⁹ The present results suggest that expression of ApoE4 in glial cells, secretion, and eventual uptake by neurons were not a sufficient mechanism to cause protein tau hyperphosphorylation in neurons. Expression in neurons in combination with aging appeared to be the important factors.

Ubiquitin immunoreactivity has been associated with hyperphosphorylated protein tau in AD.⁶⁰ In our experimental models, ubiquitin-positive inclusions were demonstrated in the brain of transgenic Thy1-ApoE4 and PDGF-ApoE4 mice, which were not found in Wt or GFAP-ApoE4 mice. However, silver staining or immunohistochemistry for protein tau did not reveal intraneuronal tangles or abnormal inclusions. In agreement with these results, neurofibrillary tangle formation could not be demonstrated in mouse models showing hyperphosphorylation of protein tau, not even when mice were 19 months old^{61–63} or in mice overexpressing and hyperphosphorylating human protein tau isoforms.^{64–66} In addition, it has been shown that AT8 immunoreactivity precedes tangle formation in AD and natural animal models such as aging sheep and goats.^{67,68}

Reactive astrocytes reflect general central nervous system injury.⁶⁹ Astroglia in the neocortex, hippocampus, and amygdala was strongest in Thy1-ApoE4, PDGF-ApoE4, and PGK-ApoE4 mice and absent in Wt mice. Only mild astroglia was present in GFAP-ApoE4 mice, and, despite higher expression levels than in the Thy1-ApoE4 mice, no signs of protein tau hyperphosphorylation were ever noted, at any age.

To explain the genetic association of ApoE4 to AD, two types of mechanisms have been proposed. First, ApoE could function as a "pathological chaperone," affecting clearance of β -amyloid and causing amyloid deposition.^{70–72} We obtained no evidence for the presence of amyloid plaques in any of our *ApoE4* transgenic mice, which of course might be because only endogenous mouse APP is present, which is less amyloidogenic.³⁹ The second hypothesis states that ApoE interacts with the microtubule-associated protein tau, thereby altering its phosphorylation state, and hence is involved in stabilizing the neuronal cytoskeleton.^{2,73} The results presented here support this hypothesis. However, the route by which ApoE gains access to the neuronal cytoplasm has been the major criticism against this hypothesis and

subject of much speculation. Most recently, it was shown that direct expression of ApoE in the cytosol of Neuro-2a cells is toxic.⁷⁴ The *ApoE4* transgenic mice are, however, not expected to express ApoE directly in the cytosol, and we also did not observe obvious signs of neurotoxicity. In addition, the hyperphosphorylation of protein tau was not restricted to brain regions expressing human ApoE as shown by immunohistochemistry. Therefore, we favor a mechanism involving an indirect interaction between ApoE and protein tau. In addition, this implies that hyperphosphorylation of protein tau is not simply a nonspecific downstream event marking degeneration.

Although great caution must be taken in extrapolating findings from transgenic mice to complex human neurodegenerative diseases such as AD, the results presented here might be relevant for the pathological process in AD. An obvious question is whether expression of the ApoE3 isoform in neurons, to the same levels as ApoE4, would have the same or other effects. Reconciling two different hypotheses, we propose that the phenotype we observed is not typical for ApoE4, but will be observed with all ApoE isoforms. Recent *in situ* hybridization experiments on human brain tissue and brain tissue of humanized transgenic lines carrying genomic sequences for ApoE demonstrated that low levels of human ApoE are expressed in neurons, independently of the ApoE isotype,^{20–23} suggesting that neuronal synthesis of ApoE is typical for and caused by regulatory sequences in the human *ApoE* gene. In addition, polymorphisms in the promoter region of the *ApoE* gene have been associated with AD,^{7–10,75–77} and many of them were correlated with increased ApoE expression.^{10,11,76,78} It is important that tight linkage of *ApoE* gene promoter polymorphisms with the ϵ 4 allele was demonstrated.^{7,8,79} Although direct evidence is lacking at this moment, combined with our observation that neuronal expression of ApoE results in hyperphosphorylation of protein tau, it is tempting to speculate that quantitative differences in ApoE expression in neurons might be related to promoter polymorphisms and that altered neuronal expression of ApoE would be genetically associated with the ϵ 4 allele. This not only points to an important function for neuronal ApoE but could also provide an alternative explanation for the observed association of the ϵ 4 allele of *ApoE* with AD. To prove this last hypothesis, a quantitative analysis is needed of ApoE expression in neurons, related to the different ApoE promoter polymorphisms and different *ApoE* alleles in AD and controls. Obviously, this hypothesis does not exclude additional effects, ie, a different extracellular role of the different ApoE isoforms on neurons, as indicated by cell culture experiments.^{31–37,80–82}

In conclusion, we demonstrated that expression of human ApoE4 in neurons resulted in hyperphosphorylation of the microtubule-associated protein tau *in vivo*, as opposed to expression in non-neuronal cells. We also showed that the neuronal expression levels of human ApoE, as well as aging, are major factors for hyperphosphorylation of protein tau to become evident. Although many different aspects still need to be addressed, as

discussed, the current transgenic mice offer the opportunity to investigate the interactions between ApoE and protein tau.

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