*Articles*

# Generation and Characterization of Two Transgenic Mouse Lines Expressing Human ApoE2 in Neurons and Glial Cells†

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ABSTRACT: Apolipoprotein E (apoE) isoforms are key determinants of susceptibility to late-onset Alzheimer's disease (AD). The $\epsilon$4 and $\epsilon$2 alleles have been associated with increased and decreased risk for AD, respectively. We have generated and characterized transgenic mice in which the human apoE2 gene is expressed under the control of the platelet-derived growth factor B-chain (PDGF-B) promoter, or the transferrin (TF) promoter. S1 nuclease analysis and immunoblotting showed that the PDGF-B apoE2 mice express apoE2 exclusively in the brain whereas the TF apoE2 mice express apoE2 in the liver and in the brain. In the TF apoE2 mouse line, apoE2 is also detected in the plasma. The PDGF-B apoE2 and the TF apoE2 transgenic mice were bred back to apoE$^{-/-}$ background. Immunohistochemical analysis showed that the PDGF apoE2 × apoE$^{-/-}$ and the TF apoE2 × apoE$^{-/-}$ mice express human apoE2 within the neocortex in hippocampal neurons and glial cells, respectively. ApoE$^{-/-}$ mice have been shown to develop age-dependent loss of synaptophysin. Immunoblotting of mouse brain extracts and immunohistochemical analysis of brain sections showed that apoE expression in both apoE2 × apoE$^{-/-}$ transgenic lines was associated with significant recovery of brain synaptophysin levels as compared to the levels of apoE$^{-/-}$ littermates of the same age. These apoE2-expressing mice, when bred back on amyloid precursor protein (APP) transgenic mice or other mouse lines featuring alterations in lipoprotein metabolism, may provide new mouse models for elucidating the role of apoE2 in lipid homeostasis in the brain and in the pathogenesis of AD.

ApoE[1] is a 34.2 kDa protein involved in the transport and homeostasis of cholesterol and other lipids (*1*). ApoE is the first known susceptibility gene for the common late-onset form of Alzheimer's disease (AD) (*2*, *3*). Human apoE exists in three major isoforms: E2 (Cys 112−Cys 158), E3 (Cys 112−Arg 158), and E4 (Arg 112−Arg 158) encoded by three common apoE alleles (*4*). Epidemiological studies have shown that the $\epsilon$4 allele increases the risk of AD while the $\epsilon$2 allele may have a protective effect (*5*−*7*). ApoE has also been implicated as a risk factor in head trauma (*8*, *9*) cerebral hemorrhage (*10*), and stroke (*11*).

ApoE is synthesized by the liver and most of the peripheral tissues, and by the central nervous system (CNS) (*12*−*14*). ApoE within the CNS is synthesized primarily by astrocytes (*13*, *14*) and microglia (*15*). ApoE is also synthesized by neurons in the human hippocampus and frontal cortex (*16*). Earlier in vitro studies have indicated that lipid-free apoE and apoE-containing lipoproteins may modulate neurite outgrowth and branching (*17*−*19*). In vivo studies have shown that the density of synaptophysin immunoreactive nerve terminals of apoE$^{-/-}$ mice showed an age-dependent decline compared to the density of apoE$^{+/+}$ mice of the same age (*20*). Also, apoE$^{-/-}$ mice when tested in a water maze performed poorly compared to the apoE$^{+/+}$ mice (*21*). Expression of the human apoE3 isoform in the CNS of apoE$^{-/-}$ mice has been shown to confer protection against age-related and endotoxin-induced neurodegeneration, whereas apoE4 expression does not confer protection (*22*−*24*).

To study the in vivo functions of apoE in the brain, we have generated transgenic mice that express human apoE2 in the brain under the control of 1.4 kb of the PDGF-B or 0.69 kb of the TF promoter. It was shown previously that the PDGF-B promoter directs gene expression in neurons (*25*) while the TF promoter directs the expression mainly in glial cells and to a lesser extent in neurons (*26*). Two transgenic mouse lines with similar levels of expression of

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[1] Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; apoE, apolipoprotein E; ABCA1, ATP-binding cassette A1 transporter; CNS, central nervous system; GFAP, glial acidic fibrillary protein promoter; PLP, paraformaldehyde; PBS, phosphate-buffered saline; PDGF-B, platelet-derived growth factor B-chain; TF, transferrin.

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Table 1: Oligonucleotides Used in PCR Amplification

| | | |
|---|---|---|
| PDGF *Xba*I | **ACTAT**TCTAGA**GGATCCACAGTCTCCTGAGTAGCTG** | PDGF −1360/−1325 sense containing an *Xba*I site |
| PDGF *Xho*I | **TGCTA**CTCGAG**CTAGGGAGGCAGCGGGGGAGGC** | PDGF +43/+75 antisense containing an *Xho*I site |
| TF *Hin*dIII | **ACCC**AAGCTT**GGTACCTAGCTTGAGGGCGGGAAGTTTTCC** | transferrin −621/−598 sense containing a *Hin*dIII site and a *Kpn*I site |
| TF *Xho*I | **TCCG**CTCGAG**CTTCCGGGTGCGGCGCTGAGCAG** | transferrin +24/+64 antisense containing an *Xho*I site |

human apoE2 were selected. The apoE2-expressing mice were bred back on the apoE$^{-/-}$ background to generate transgenics in the apoE$^{-/-}$ background. Expression of the human apoE2 transgene was established by RNA and protein analysis in the brain of the transgenic mice. Brain organ cultures showed that apoE2 could be secreted into the culture medium. Immunoblot analysis of brains from 8-month-old mice showed a significant reduction in synaptophysin levels in the brains of the apoE$^{-/-}$ mice compared with the levels in the wild-type apoE$^{+/+}$ mice. Consistent with previous reports, the brains of the PDGF apoE2 × apoE$^{-/-}$ and TF apoE2 × apoE$^{-/-}$ mice of the same age showed a significant recovery of brain synaptophysin levels compared to the levels of the apoE$^{-/-}$ mice.

Future analysis of these mice and derivatives generated by breeding with the appropriate mouse models of AD and lipoprotein metabolism may provide new insights into the contribution of apoE2 in either the protection from or the pathogenesis of AD.

## EXPERIMENTAL PROCEDURES

### *Materials*

Reagents were purchased from the following sources: restriction and modifying enzymes from New England Biolabs (Beverly, MA), [$\gamma$-$^{32}$P]ATP (5000 Ci/mmol), [$\alpha$-$^{32}$P]-dCTP, and [$\alpha$-$^{32}$P]dGTP (3000 Ci/mmol) from New England Nuclear (Boston, MA), bactotryptone and bacto yeast extracts from Difco (Detroit, MI), acrylamide, sodium dodecyl sulfate (SDS), urea, and Tris from International Biotechnologies, Inc. (Rochester, NY), bacterial DH5$\alpha$ cells from Stratagene (La Jolla, CA), custom-made oligonucleotides from Gibco BRL (Rockville, MD), plasmid pSPORT-1 from Gibco BRL, S1 nuclease from Boehringer Mannheim (Indianapolis, IN), and proteinase K, spermine, phenylmethanesulfonyl fluoride (PMSF), and ammonium acetate from Sigma (St. Louis, MO). The mouse apoE cDNA clone was purchased from ATCC. C57Bl6 inbred and Swiss Webster outbred mice were purchased from Taconic Farms. ApoE$^{-/-}$ knockout mice were purchased from Jackson Laboratories. Transgenic mice were generated in the transgenic facility of the Boston University Laboratory Animal Science Facility. The monoclonal anti-human apoE antibody 3H1 was obtained from the Ottawa University Lipoprotein Core Facility. Two different antibodies were used to detect mouse synaptophysin. A rabbit anti-synaptophysin polyclonal antibody from Calbiochem (San Diego, CA) was used for Western blotting, and a rabbit anti-human synaptophysin antibody from DACO was used for immunohistochemistry.

### *Methods*

*Preparation of the Gene Construct Used in the Generation of Transgenic Mice.* The human PDGF−apoE2 gene construct used for microinjection into mouse fertilized oocytes was prepared as follows: a 1.4 kb fragment of the psisCAT6a plasmid, containing the human PDGF-B chain promoter (kindly provided by T. Collins), was amplified using the PDGF−*Xba*I sense oligo and the PDGF−*Xho*I antisense oligo, which contained *Xba*I and *Xho*I sites at the 5′ and 3′ termini, respectively. The 1.4 kb PCR product was digested with *Xba*I and *Xho*I and cloned into the corresponding sites of a pBluescript SK$^{+/-}$ vector (Stratagene). This pBluescript SK$^{+/-}$ plasmid containing the 1.4 kb PDGF-B promoter was digested with *Xho*I, and a 4.9 kb *Xho*I−*Xho*I fragment of the human apoE2 gene containing exons 1−4 was cloned in this plasmid 3′ to the PDGF promoter. The correct orientation was confirmed by *Xba*I digestion. The pBluescript SK$^{+/-}$ plasmid containing the PDGF−apoE2 construct was digested with *Xba*I and *Kpn*I and cloned into the respective sites of the pSPORT vector. The correct orientation was confirmed with *Xho*I digestion. The PDGF−apoE2 construct was excised from the pSPORT vector following digestion with *Kpn*I, and *Hin*dIII was separated on a 1% low-melting point agarose gel, purified with $\beta$-agarose enzyme (NEB), and diluted in TE (10 mM Tris-HCl and 0.1 mM EDTA) buffer to a final concentration of 3 $\mu$g/mL.

The human TF−apoE2 gene construct used for microinjection into mouse fertilized oocytes was prepared as follows: a human genomic DNA from HepG2 cells was used to generate by PCR amplification the 1−693 bp region of the TF promoter, using a 5′ TF−*Hin*dIII sense oligo and a 3′ TF−*Xho*I antisense oligo as primers which contained *Xho*I and *Hin*dIII sites at the 5′ and 3′ termini, respectively (Table 1). The 0.69 kb PCR product was digested with *Hin*dIII and *Xho*I and cloned into the corresponding sites of a pBluescript SK$^{+/-}$ vector. Following digestion of the pBluescript SK$^{+/-}$ vector containing the 0.69 kb TF promoter with *Xho*I, a 4.9 kb *Xho*I−*Xho*I fragment of the human apoE2 gene containing exons 1−4 was cloned into this plasmid 3′ to the TF promoter. The correct orientation was confirmed with restriction digestion with *Xho*I. The TF−apoE2 construct was excised from the pBluescript vector by digestion with *Kpn*I and *Hin*dIII, separated on a 1% low-melting point agarose gel, purified with $\beta$-agarose enzyme, and diluted in TE buffer to a final concentration of 3 $\mu$g/mL.

*Generation of Transgenic Mice.* Original inbred C57BL/6 and outbred Swiss Webster mice were obtained from Taconic Farms and maintained in the transgenic facility of the Laboratory of Animal Science at Boston University Medical Center. To obtain fertilized oocytes for microinjection, 3−4-week-old C57BL/6 female mice were superovulated as described previously (*27*) and mated with C57BL/6 males. Fertilized oocytes were collected the next morning and used for pronuclear injection. Viable oocytes were transferred the same day in Swiss Webster pseudopregnant females and allowed to develop to term. To identify transgenic founder mice, 10 $\mu$g of genomic DNA was isolated from tail biopsies at 20 days of age, digested with *Eco*RI, and analyzed by

Southern blot hybridization as described previously (*28*), using human apoE2 cDNA as a DNA probe. Transgenic progeny from the founder mice were identified by either Southern or slot blot hybridization analysis.

*Breeding of the Human PDGF-B ApoE2 Transgenic Mice on the ApoE$^{-/-}$ Background and Screening of the Knockout Mice.* Heterozygote transgenic mice (C57BL/6) for the human PDGF-B apoE2 or the TF apoE2 gene were mated with apoE$^{-/-}$ mice (129 Ola) obtained from Jackson Laboratories. From the F1 generation (50% C57BL/6 and 50% 129 Ola), heterozygote human PDGF−apoE2 or TF−apoE2 transgenic mice that were apoE$^{+/-}$ were selected and backcrossed to apoE$^{-/-}$ mice. From the F2 generation (25% C57BL/6 and 75% 129 Ola), heterozygote human PDGF−apoE2 (or TF−apoE2) transgenic mice that were apoE$^{-/-}$ were mated with apoE$^{-/-}$ mice. From the F3 generation (12.5% C57BL/6 and 87.2% 129 Ola), heterozygote human PDGF-B−apoE2 or TF−apoE2 transgenic mice were kept for the study, and their nontransgenic apoE$^{-/-}$ littermates were kept as controls. Also, wild-type mice (129 Ola), obtained from Harlan, of the same age were kept under the same conditions in the same room as controls. To screen for the presence of the mouse apoE endogenous gene, DNA extracted from tail biopsies was amplified by PCR using the following primers: apoE5, 5′-GCCTAGCCGAGGGAGAGC-CG-3′; apoE3, 5′-TGTGACTTGGGAGCTCTGCAGC-3′; and Neo3, 5′-GCCGCCCCGACTGCATCT-3′. ApoE5 and apoE3 amplify a 155 bp wild-type mouse apoE gene, and the apoE5 and Neo3 primers amplify a 245 bp fragment of the modified mouse apoE gene containing the Neo insert. Mice containing only the 245 bp fragment represent the apoE2 × E$^{-/-}$ transgenic mice.

*RNA Extraction and S1 Nuclease Protection Analysis.* Total RNA was extracted from dissected mouse tissue using the LiCl/urea method (*27*). Freshly collected tissues were homogenized and sonicated in LiCl/urea buffer. The following day, samples were centrifuged, pellets were resuspended in TES (100 mM Tris, 10 mM EDTA, and 1% SDS) buffer, and an equal volume of a phenol/chloroform/isoamyl alcohol mixture (25:24:1) was added. The RNA in the supernatant was precipitated with 2.5 volumes of 100% ethanol and $^1/_{10}$ volume of 3 M sodium acetate (pH 5.3). RNA pellets were redissolved in TE (Tris EDTA) buffer. S1 nuclease protection was performed as described previously (*29*). Briefly, 20 $\mu$g of total RNA was resuspended in S1 hybridization buffer [40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA, and 80% formamide] and hybridized at 52 °C overnight with human apoE2 and a mouse $\beta$-actin specific probe. The next day, 300 $\mu$L of digestion buffer [280 mM NaCl, 30 mM sodium acetate, and 4.5 mM zinc acetate (pH 4.8)] containing S1 nuclease enzyme was added in the hybridization mix and kept at room temperature for 2 h. An equal volume of a phenol/chloroform/isoamyl alcohol mixture (25:24:1) was then added, and the supernatant was precipitated with 2.5 volumes of ethanol. Pellets were resuspended in loading buffer and were separated on a 6% acrylamide−urea gel. The gel was exposed overnight to X-ray film. A human *Kpn*I−*Bbs*I apoE DNA fragment containing 859 bp of full-length apoE cDNA (*30*) along with 667 bp of the CMV promoter was used as a probe for apoE. A mouse *Pvu*II apoE DNA fragment containing 300 bp from pBluescript and 923 bp from mouse apoE cDNA was used as a probe for mouse apoE. The actin probe represented a 240 bp *Ava*I−*Bam*HI restriction fragment of the $\beta$-actin gene and contained 100 bp of exon I and 140 bp of the 5′ end of the gene (*29*).

*Western Blotting and Immunodetection.* Brain samples were homogenized in suspension buffer containing 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.6), 0.001 M EDTA (pH 8.0), 1 $\mu$g/mL aprotinin, and 100 $\mu$g/mL PMSF for the detection of human apoE2 (*28*) or 0.02 M NaCl, 0.01 M Tris-HCl (pH 7.6), 0.01 M EDTA (pH 8.0), 0.01 M EGTA (pH 7.6), 1% SDS, 1 $\mu$g/mL aprotinin, and 100 $\mu$g/mL PMSF for the detection of mouse synaptophysin. Insoluble material was removed by centrifugation, and an equal volume of 2× SDS gel-loading buffer was added to the supernatant, after which the samples were boiled for 10 min. Samples were loaded on a 10% SDS−acrylamide gel, separated by SDS−PAGE, and electrotransferred onto Immobilon-P membranes (Millipore). The membranes were blocked in TBS containing 5% nonfat dried milk for the detection of human apoE2 or 10% fetal calf serum for the detection of mouse synaptophysin, and subsequently incubated with the 3H1 anti-human apoE mouse monoclonal antibody (1:2000) or the rabbit anti-mouse synaptophysin polyclonal antibody (1:500). The bound primary antibody was detected with HRP-bound goat anti-mouse IgG (Boehringer Mannheim) (1:1000) for human apoE2 or anti-rabbit IgG for mouse synaptophysin (1:10000). Immunodetection was performed with ECL reagents according to the manufacturer's protocol (Amersham) and quantitated by scanning densitometry. Two independent experiments were performed in duplicate.

*Brain Organ Cultures.* Freshly dissected brain tissue was removed and cut into very small pieces (0.2−1 mm) in DMEM and maintained in a Torbal organ culture jar in 95% $O_2$ and 5% $CO_2$ for 6 h. The culture media were collected and concentrated 30-fold using Amicon centrifugal filter devices (Centricon YM-10) and analyzed by SDS−PAGE, transferred onto nitrocellulose membrane, and probed with a human apoE monoclonal antibody, as described above. The culture medium of HepG2 cells that secrete human apoE protein was used as a positive control.

*Immunohistochemistry for Human ApoE2.* For this analysis, mice were perfused transcardially with 0.1 M phosphate buffer (pH 7.4), followed by a periodate/lysine/2% paraformaldehyde (PLP) solution. Brain tissue was removed and kept overnight in the PLP solution at 4 °C. The next day, the PLP solution was replaced with 10% glycerol, 2% DMSO, and 0.1 M phosphate buffer (pH 7.4). The following day, the solution was replaced with 20% glycerol and 2% DMSO in 0.1 M phosphate buffer (pH 7.4), and the tissue was kept in this solution until it was processed. Fixed tissues were cut in 50 $\mu$m thick sections on a sliding microtome and processed for analysis of peroxidase immunohistochemistry. Sections were incubated in 0.3% $H_2O_2$ and methanol for 30 min to quench endogenous peroxidase activity and washed for 10 min in 0.1 M PBS (three times). After incubation for 1 h in 10% normal goat serum in 0.1 M PBS, sections were treated with anti-human apoE (Chemicon International Inc.) diluted 1:100 in 2% normal goat serum in 0.1 M PBS, for 1 h. Sections were then rinsed three times in 0.1 M PBS for 10 min and incubated with the secondary antibody diluted 1:1000 (peroxidase-conjugated goat anti-mouse IgG, from Boehringer Mannheim) in 2% normal goat serum for 3 h.

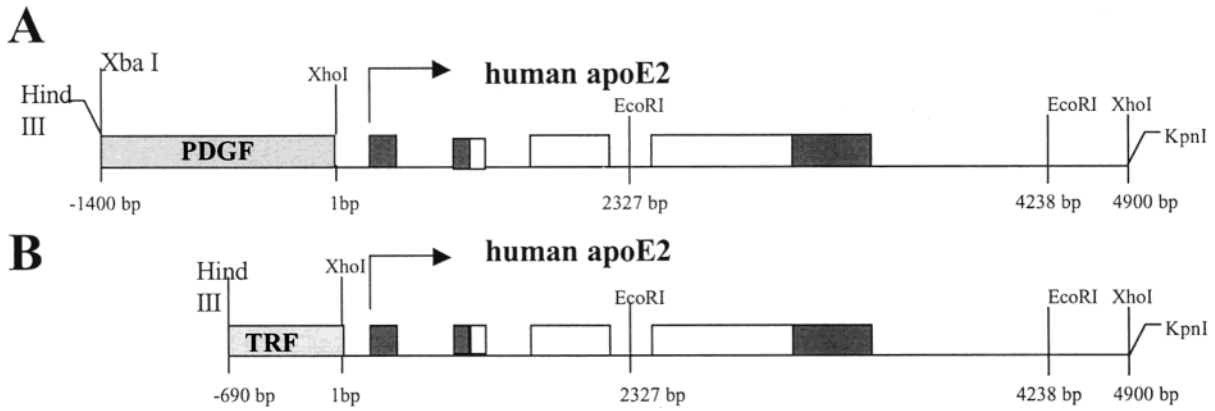


FIGURE 1: ApoE2 gene constructs used for the generation of transgenic mice. (A) The PDGF promoter (1.4 kb) was placed 5′ to a promoterless 4.9 kb human apoE2 gene to direct expression in neurons. (B) The TF promoter (0.69 kb) was placed 5′ to a 4.9 kb promoterless human apoE2 gene to direct expression in glial cells.

Sections were again rinsed three times in 0.1 M PBS for 10 min and incubated in a solution containing 0.5 mg/mL diaminobenzidine tetrahydrochloride and 0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.6) containing imidazole [110 mL of 0.05 M Tris buffer (pH 7.6) mixed with 10 mL of 0.1 M phosphate buffer (pH 7.6) containing 0.7 g of imidazole] for 5−10 min. Finally, sections were rinsed three times in 0.1 M PBS for 10 min, dried, and then dehydrated and cover slipped with Permount.

*Immunohistochemistry for Mouse Synaptophysin.* Brains were removed from perfused mice as described above, fixed, and cut on a sliding microtome in 50 $\mu$m thick sections. Sections were treated as described above and processed for analysis of peroxidase immunohistochemistry. Synaptophysin was detected with a rabbit anti-human synaptophysin antibody (DACO A/S Denmark) diluted 1:100 that also detects mouse and rat synaptophysin. As a secondary antibody, an anti-rabbit IgG (H+L)−alkaline phosphatase conjugate (Boehringer Mannheim) diluted 1:1000 was used.

*Quantification of Human ApoE.* Serum human apoE concentrations were measured using a sandwich ELISA (*31*). Affinity-purified polyclonal goat anti-human apoE antibodies were used for coating microtiter plates, and polyclonal goat anti-human apoE coupled to horseradish peroxidase was used as the secondary antibody. The immunoperoxidase procedure was employed for the colorimetric detection of apoE at 450 nm, using tetramethylbenzidine as the substrate. Pooled plasma from healthy human subjects with known apoE levels was used as a standard.

*Triglyceride and Cholesterol Analysis.* The serum sample (10 $\mu$L) was diluted with 40 $\mu$L of phosphate-buffered saline (PBS), and 7.5 $\mu$L of the dilute sample was analyzed for triglycerides and cholesterol using the GPO-Trinder Kit (Sigma) and the CHOL-MPR3 kit (Roche Molecular Biochemicals), according to the manufacturer's instructions. Triglyceride and cholesterol concentrations were determined spectrophotometrically at 540 and 492 nm, respectively.

## RESULTS

*Generation and Characterization of Transgenic Mice Carrying the Human PDGF−ApoE2 or the Human TF−ApoE2 Gene Construct in the ApoE$^{-/-}$ Background.* Two apoE2 gene constructs, PDGF−apoE2 and TF−apoE2, were used to generate transgenic mice. In the first construct, the human apoE2 gene sequence containing all four exons was linked to the PDGF-B chain promoter that directs gene expression in the neurons (*25*) (Figure 1A). In the second construct, the human apoE2 gene sequence containing all four exons was linked to the human TF promoter that directs expression in the glial cells (*26*) (Figure 1B). Two mouse lines that had comparable levels of human apoE2 expression in the brain (one from each construct; Figure 1A,B) were used for further studies and crossed onto apoE$^{-/-}$ background as described in Experimental Procedures. Southern blotting analysis of genomic DNA obtained from transgenic mice confirmed the structural integrity of the transgenes. Transgenic mice developed normally and showed no macroscopic difference compared to their nontransgenic littermates.

*Tissue Specific Distribution of ApoE2 mRNA.* The expression patterns of the human apoE2 mRNA were examined by S1 nuclease protection analysis of total RNA as described in Experimental Procedures (Figure 2A,B). High levels of apoE2 mRNA were detected in the brain of both PDGF-B apoE2 × apoE$^{-/-}$ and TF apoE2 × apoE$^{-/-}$ mice as reported previously (Figure 2A,B) (*25*, *26*). In addition, high levels of human apoE2 mRNA were also detected in the liver of the TF apoE2 × apoE$^{-/-}$ mice (Figure 2A). Human apoE mRNA could not be detected in the kidney or intestine in either of the transgenic lines. A quantitative S1 nuclease protection analysis was also performed using total RNA isolated from brains of PDGF apoE2 × apoE$^{-/-}$ and nontransgenic mice or TF apoE2 × apoE$^{-/-}$ and nontransgenic mice. An aliquot of 10 $\mu$g of total RNA was hybridized with probes having 40 000 cpm of human apoE2 and 40 000 cpm of mouse apoE, as described in Experimental Procedures. A mouse $\beta$-actin specific probe (40 000 cpm) was used as a control to assess differences between RNA preparations. This analysis showed that the steady-state levels of human apoE2 mRNA are much greater (approximately 30-fold increase) than the mouse apoE mRNA levels (Figure 2B).

*The Human ApoE2 Protein Is Synthesized and Secreted in the Brains of PDGF ApoE2 × ApoE$^{-/-}$ and TF ApoE2 × ApoE$^{-/-}$ Mice.* Western blotting analysis of homogenates of brain tissue from both lines showed the presence of the human apoE2 protein in the brain homogenates of PDGF apoE2 × apoE$^{-/-}$ and TF apoE2 × apoE$^{-/-}$ transgenic mice (Figure 3A). The human apoE2 protein was also detected in the liver homogenates of TF apoE2 × apoE$^{-/-}$ transgenic

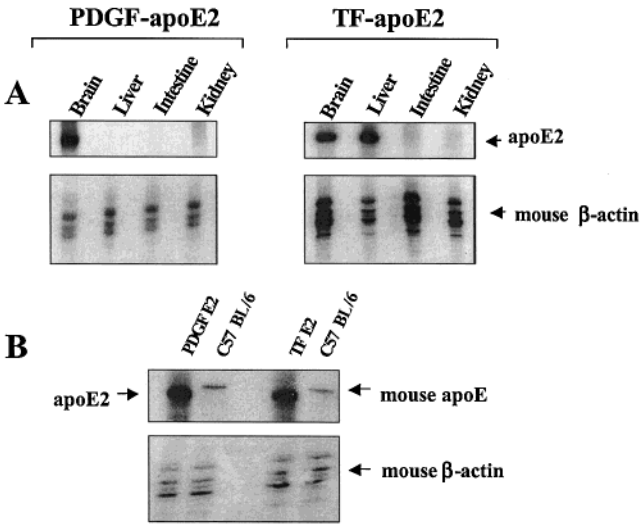


FIGURE 2: S1 nuclease protection analysis of total RNA isolated from tissues of F1 mice carrying the human PDGF−apoE2 transgene or the TF−apoE2 transgene. (A) Ten micrograms of total RNA isolated from brain, liver, intestine, and kidney was hybridized to analyze expression for hu-apoE2 and mouse $\beta$-actin. The probes used and the S1 nuclease analysis are described in Experimental Procedures. (B) S1 nuclease protection analysis of total RNA isolated from brains of PDGF−apoE2, TF−apoE2, and wild-type C57BL/6 mice. Ten micrograms of total RNA isolated from brain was hybridized as indicated for panel A. The tissues analyzed for apoE mRNA synthesis are shown on the top of panels A and B. The human apoE2, mouse apoE, and $\beta$-actin mRNA bands corresponding to 859, 923, and 100 nucleotides, respectively, are indicated.

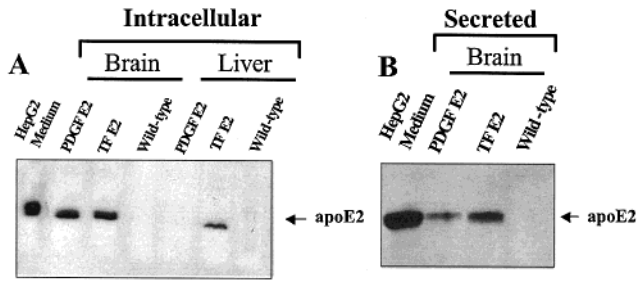


FIGURE 3: Immunoblotting of human apoE2 in whole brain and liver homogenates or organ culture media of transgenic mice expressing the PDGF−apoE2 or TF−apoE2 construct and wild-type control mice. Brains from PDGF−apoE2 Tg mice, TF−apoE2 Tg mice, and wild-type mice were removed and cut into very small pieces (0.2−1 mm) in DMEM and maintained in a Torbal organ culture jar in 95% $O_2$ and 5% $CO_2$ for 6 h. (A) Supernatants from brain and liver homogenates from PDGF−apoE2, TF−apoE2, and wild-type mice were analyzed by 10% SDS−PAGE, transferred onto a nitrocellulose membrane, and probed with a human apoE monoclonal antibody as explained in Experimental Procedures. (B) Aliquots of 15 $\mu$L of organ culture medium concentrated 30-fold were analyzed by 10% SDS−PAGE, transferred onto a nitrocellulose membrane, and probed with a human apoE monoclonal antibody as described in Experimental Procedures. The origins of the samples that were analyzed are indicated at the top of panels A and B. The culture medium of HepG2 cells, which secrete human apoE protein, was used as a positive control in both panels.

mice (Figure 3A). In these mice, apoE2 was also detected in plasma. Organ cultures of brain tissue from PDGF apoE2 $\times$ apoE$^{-/-}$ and TF apoE2 $\times$ apoE$^{-/-}$ transgenic mice showed that the protein that is produced is secreted into the culture medium (Figure 3B). This finding establishes that apoE synthesized by neurons in PDGF apoE2 $\times$ apoE$^{-/-}$ Tg mice and glial cells in TF apoE2 $\times$ apoE$^{-/-}$ transgenic mice is

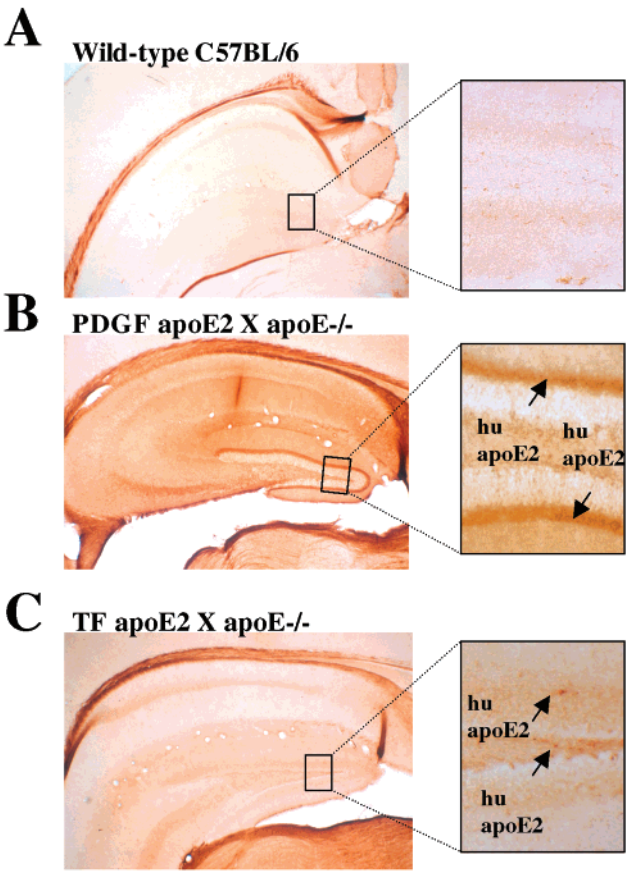


FIGURE 4: Detection of apoE immunoreactivity in sections of the hippocampus of PDGF apoE2 $\times$ apoE$^{-/-}$ and TF apoE2 $\times$ apoE$^{-/-}$ Tg mice. Brain sections of 8-month-old wild-type C57Bl/6 control (A), PDGF apoE2 $\times$ apoE$^{-/-}$ (B), and TF apoE2 $\times$ apoE$^{-/-}$ (C) mice of the same age were analyzed by immunocytochemistry as described in Experimental Procedures. In the PDGF−apoE2 sections, the observed strong neuronal expression of human apo-E is indicated by arrows (B); in the TF−apoE2 sections, the expression of the human apoE is indicated by arrows. The diffused pattern of expression in panel C is compatible with expression in glial cells.

secreted in the brain parenchyma, where it can exert its physiological functions.

*Plasma Cholesterol, Triglyceride, and Human ApoE Levels.* The average plasma lipids and apoE levels were analyzed in a group of four to six mice ages 7−8 months. The triglycerides levels were in the range of 140−160 mg/dL and did not differ significantly from the triglyceride levels of the apoE$^{-/-}$ mice. The average cholesterol level of the apoE$^{-/-}$ mice was 934 $\pm$ 217 mg/dL and was reduced by 58% in the TF apoE2 $\times$ apoE$^{-/-}$ mice. ApoE at concentrations of 6.2 $\pm$ 3.7 $\mu$g/mL was detected only in the plasma of TF apoE2 $\times$ apoE$^{-/-}$ mice.

*Human ApoE2 Can Be Localized in Brain Sections of PDGF ApoE2 $\times$ ApoE$^{-/-}$ and TF ApoE2 $\times$ ApoE$^{-/-}$ Mice.* Immunohistochemical analysis of brain sections with a specific human apoE antibody did not detect human apoE immunoreactivity in the brain of wild-type control mice (Figure 4A). In contrast, immunohistochemical analysis of PDGF apoE2 $\times$ apoE$^{-/-}$ transgenic mice showed widespread expression of the human apoE2 protein in hippocampus with intense staining in neurons (Figure 4B). Immunohistochemical analysis of similar brain sections of TF apoE2 $\times$ apoE$^{-/-}$ transgenic mice showed expression of the human apoE2 protein throughout the brain, most likely indicating expres-

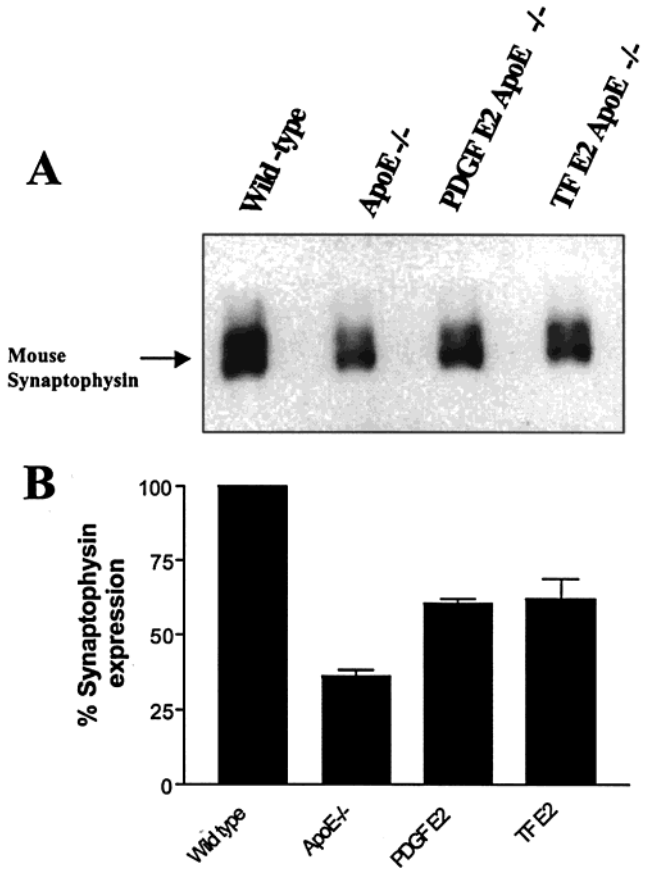


FIGURE 5: (A) Immunoblotting analysis of total protein extracts obtained from brain homogenates of PDGF apoE2 × apoE$^{-/-}$, TF apoE2 × apoE$^{-/-}$, apoE$^{-/-}$, and wild-type mice. Equal amounts of brain homogenates were analyzed by 10% SDS−PAGE, transferred onto a nitrocellulose membrane, and hybridized with a rabbit anti-synaptophysin polyclonal antibody (Calbiochem). The origins of the samples are indicated at the top of the figure. (B) Intensities of the bands of panel A, as assessed by scanning densitometry. The difference between synaptophysin levels in apoE$^{-/-}$ mice and PDGF apoE2 × apoE$^{-/-}$ and TF apoE2 × apoE$^{-/-}$ mice were significant at $p < 0.001$ and $p < 0.03$, respectively, based on Student's $t$ tests.

sion in glial cells. The staining of apoE2 in the TF apoE2 × apoE$^{-/-}$ transgenic mice was less abundant than that in the PDGF apoE2 × apoE$^{-/-}$ transgenic mice (Figure 4C).

*Mouse Synaptophysin Expression Is Partially Restored in the PDGF ApoE2 × ApoE$^{-/-}$ and TF ApoE2 × ApoE$^{-/-}$ Transgenic Mice.* Western blotting analysis of the protein extract of brain tissue obtained from wild-type nontransgenic, apoE$^{-/-}$, PDGF apoE2 × apoE$^{-/-}$, and TF apoE2 × apoE$^{-/-}$ mice showed that synaptophysin levels were reduced by approximately 62% in apoE$^{-/-}$ mice compared to the wild-type controls (Figure 5A,B). This finding is consistent with previous findings (*20*). The same analysis also showed that synaptophysin levels increased significantly (by approximately 60%) in the PDGF apoE2 × apoE$^{-/-}$ and TF apoE2 × apoE$^{-/-}$ mice compared to the level in apoE$^{-/-}$ mice (Figure 5A,B). Synaptophysin levels were also analyzed by immunohistochemistry in brain sections of wild-type, apoE$^{-/-}$, PDGF apoE2 × apoE$^{-/-}$, and TF apoE2 × apoE$^{-/-}$ mice. Wild-type mice, as expected, exhibited strong expression of synaptophysin immunoreactivity (Figure 6A), while apo E$^{-/-}$ mice exhibited a significant reduction in the level of synaptophysin staining (Figure 6B). In comparison, PDGF

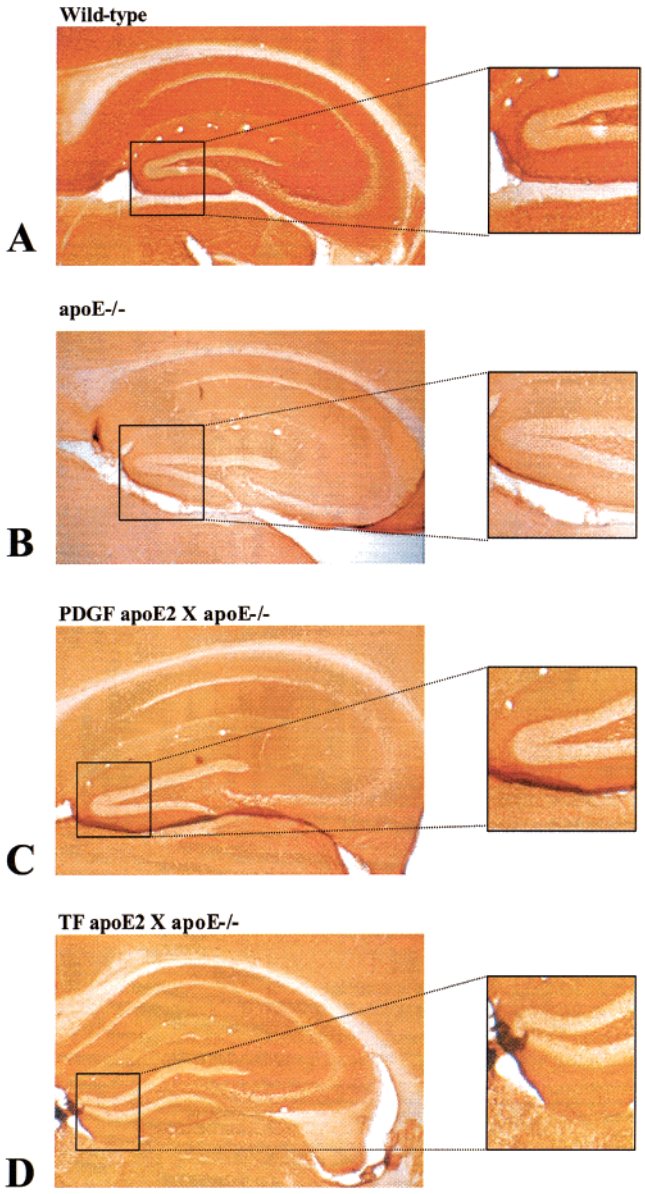


FIGURE 6: Detection of synaptophysin immunoreactivity. Hippocampal brain sections of PDGF apoE2 × apoE$^{-/-}$ and TF apoE2 × apoE$^{-/-}$ transgenic and control mice. Eight-month-old transgenic and control mice were immunostained with an antibody against synaptophysin, which is a marker of a presynaptic terminal. Sections of hippocampus from brains of PDGF apoE2 × apoE$^{-/-}$ (C) and TF apoE2 × apoE$^{-/-}$ (D) showed stronger synaptophysin staining than brain sections of their apoE$^{-/-}$ littermates of the same age (B). The sections of wild-type nontransgenic (A) and apoE$^{-/-}$ mice (B) of the same age were used as positive and negative controls, respectively.

apoE2 × apoE$^{-/-}$ and TF apoE2 × apoE$^{-/-}$ mice of the same age exhibited intermediate levels of synaptophysin immunoreactivity (Figure 6C,D). Thus, the immunohistochemical analysis is consistent with the immunoblotting analysis and suggests that expression of human apoE2 restored to some extent synaptophysin levels in the brain of apoE$^{-/-}$ mice.

## DISCUSSION

ApoE has been implicated in both cardiovascular disease and AD (*1*). Epidemiological studies have established that the $\epsilon$4 allele predisposes carriers to late-onset familial AD (*5*, *7*), whereas the $\epsilon$2 allele may confer protection (*6*). In

the mouse brain, apoE seems to be important in maintaining the integrity of the CNS during aging (*20*). Learning deficits have been reported in apoE-deficient mice (*32*). Various transgenic mice have been generated where the human $\epsilon$3 or $\epsilon$4 alleles are expressed in the CNS of apoE-deficient mice under specific promoters (*21*, *22*, *24*, *26*). These studies using biochemical (*22*, *24*, *26*) or behavioral data (*21*) suggested that apoE4 is associated with neurodegeneration and learning and memory deficits, symptoms that have been observed in Alzheimer's disease. In another study, the human $\epsilon$2 allele (genomic or cDNA) has been expressed in the brains of apoE$^{-/-}$ transgenic mice using brain specific promoters such as the glial acidic fibrillary protein promoter (GFAP) (*35*). Mice expressing the $\epsilon$2 allele were not studied further to evaluate the effects of human apoE2 on brain physiology or behavior (*26*, *32*).

The objective of this study was to generate and characterize human apoE2 specific transgenic mice, in apoE$^{-/-}$ background, which overexpress the protective human $\epsilon$2 allele in the brain. Consistent with the previous findings, the mice expressing $\epsilon$2 under the control of the PDGF-B promoter have high levels of apoE mRNA in neurons (*25*). This mouse line is physiologically relevant since it has been shown that neurons produce human apoE in transgenic mice which express the human apoE alleles under their own promoter (*36*). On the other hand, the TF promoter, as shown previously (*26*), expresses high levels of apoE mRNA in glial cells. In the study presented here, we established that apoE synthesized by neurons or glial cells in the brain was secreted in the brain parenchyma as assessed by the brain organ culture experiments. As discussed later, secreted lipid-free or lipid-associated apoE may participate in cholesterol efflux (*37*), whereas lipid-associated apoE may interact with cell receptors and contribute to both cholesterol delivery and cellular efflux of cholesterol (*31*−*39*). In the PDGF transgenic mice, apoE2 synthesis was confined only in the brain, and was not detected in the serum. In the TF transgenic mice, apoE2 synthesis was observed in the brain as well as in the liver, and could be detected in the serum (Figure 3B). Previous studies of TF−apoE transgenic mice using the same promoter did not provide information about the hepatic expression of apoE (*26*). Our findings indicate that the TF promoter segment that was used, contains the elements required for hepatic expression. Lipid analysis of the TF apoE2 × apoE$^{-/-}$ mice showed a 58% reduction of the plasma cholesterol levels as compared to those of apoE$^{-/-}$ mice of the same age. Immunohistochemical analysis on brain sections showed expression of human apoE2 in neuron layers in the hippocampus in the PDGF apoE2/apoE$^{-/-}$ mice. In the TF apoE2 × apoE$^{-/-}$ mice, staining was more diffused, most likely reflecting the apoE2 expression in glial cells.

To assess neurodegeneration in the PDGF and TF apoE2 × apoE$^{-/-}$ mice, we quantified the immunoreactivity for the neuronal marker synaptophysin. Loss of synaptophysin immunoreactivity has been found in AD (*40*−*43*). It has also been reported that apoE$^{-/-}$ mice exhibit a significant loss of synaptophysin immunoreactivity (*20*, *22*). Immunoblotting of total brain homogenates from PDGF apoE2 × apoE$^{-/-}$, TF apoE2 × apoE$^{-/-}$, apoE$^{-/-}$, and wild-type controls showed that synaptophysin levels were reduced by 62% in the apoE$^{-/-}$ mice as compared to the nontransgenic wild-type controls and were increased by approximately 60% in the PDGF apoE2 × apoE$^{-/-}$ and the TF apoE2 × apoE$^{-/-}$ mice, as compared to the apoE$^{-/-}$ mice. We also stained brain sections of eight-month-old apoE$^{-/-}$ mice and wild-type control mice with an anti-synaptophysin antibody. ApoE$^{-/-}$ mice showed a dramatic reduction in the level of synaptophysin staining, compared to the control mice. Staining of brain sections from PDGF and TF apoE2 × apoE$^{-/-}$ eight-month-old mice showed a significant increase in synaptophysin immunoreactivity as compared to the apoE$^{-/-}$ littermates of the same age. These data demonstrate that human apoE2 in the brain of apoE$^{-/-}$ transgenic mice can reverse partially the loss of synaptophysin, which accompanies the inactivation of the apoE gene in apoE$^{-/-}$ mice.

The reason overexpression of apoE in the brain restored only partially synaptophysin levels is not clear. It is possible though that structural differences between human and mouse apoE may directly or indirectly affect synaptophysin stability and that optimal stability may be achieved by direct or indirect interactions between apoE and synaptophysin when the two proteins are from the same species. It was shown recently that astrocytes which secrete apoE and apoE-containing lipoprotein particles increase dramatically the synaptic activity of retinal ganglion cells (*44*). These studies suggested functional interactions of apoE-containing lipoproteins with the synaptic terminals where synaptophysin resides.

Despite numerous studies, the functions of apoE in the brain which either protect against or predispose carriers to AD have not been clarified. Previous biochemical and functional data suggested isoform specific functions of apoE, including (a) binding to A$\beta$ (*45*, *46*), (b) binding to tau and MAP2C (*47*), (c) a cholinergic deficit in the frontal cortex and the hippocampus (*48*), (d) effects on neuronal morphology and cytoskeletal structure in vitro (*49*, *50*), (e) effects on neuronal degeneration and dendritic remodeling in vivo (*22*, *51*), (f) effects of A$\beta$ deposition (*52*) and plaque-associated neuritic dystrophy in transgenic mice (*52*, *53*), (g) inhibition of fusion of liposomes by apoE which is promoted by A$\beta$ (*54*), and (h) binding to soluble APP (sAPP) (*55*). Such binding enhances protection against excitotoxicity in rat hippocampal neurons and inhibits activation of microglia (*56*). The combined findings suggested that impairment of known or yet unidentified functions of apoE may play a central role in the pathogenesis of AD.

The potential role of apoE−A$\beta$ complexes in the pathogenesis of AD has been studied extensively through cell cultures, animal models, and human patients (*46*, *57*, *58*). We and others have found that the ability of natural apoE isoforms to form SDS-stable apoE−A$\beta$ complexes follows the order apoE2 > apoE3 ≫ apoE4 and correlates inversely with the risk of developing AD (*46*, *58*). A recent study showed that expression of the natural human apoE3 and apoE4 isoforms in APP-overexpressing mice reduced the level of formation of amyloid deposits to levels lower than those observed in mice which do not express their own apoE (E$^{-/-}$) (*53*, *59*). Plaque-associated neuritic dystrophy in these mice required the presence of human or mouse apoE and was 10 times higher in mice expressing apoE4 than in mice expressing apoE3 (*53*). These data provided strong evidence that apoE plays an important role in A$\beta$ deposition and plaque formation and/or in the cellular uptake of apoE−A$\beta$ complexes (*52*, *53*, *59*).

The influence of apoE isoforms on neurodegeneration was studied in mice expressing apoE3 and apoE4 in apoE$^{-/-}$ background. It was found that intraperitoneal injection of the kainic acid caused excitotoxin-induced and age-dependent neurodegeneration in apoE$^{-/-}$ mice which was corrected by the expression of apoE3 but not of apoE4 in the brain. Observed changes in apoE$^{-/-}$ mice included the loss of synaptophysin and positive presynaptic terminals as well as the loss of MAP-2 in the neocortex and the hippocampus and disruption of neurofilament-positive axons in the hippocampus. It will be interesting in future studies to determine the effect of apoE2 which protects against Alzheimer's disease on the protection of our transgenic mice from neurodegeneration (*22*).

On the other hand, apoE may also contribute to the pathogenesis of AD through various cholesterol homeostatic mechanisms. Peripheral cells derive cholesterol through endogenous cholesterol synthesis and receptor-mediated cholesterol uptake, and lose excess cholesterol through a process known as cholesterol efflux or reverse cholesterol transport (*60*). It recently became apparent that the release of cholesterol from the cell membrane is mediated by the ATP-binding cassette A1 transporter (ABCA1) (*61*). It was shown recently that apoE is one of the ligands of ABCA1 (*37*) and is capable of promoting selective uptake of cholesteryl esters (*62*) and cholesterol efflux in cell cultures (Chroni and V. I. Zannis, unpublished results). Although the expression of ABCA1 in the brain has not been established, related lipid transporters may exist (*63*, *64*). It has been established that the major acceptors of cellular cholesterol in the circulation are different HDL species, including HDL species of pre-$\beta$ electrophoretic mobility (*60*). Furthermore, it was found that HDL particles with $\gamma$ electrophoretic mobility named $\gamma$LpE, found in the plasma of apoA-I deficient mice expressing human apoE (*38*, *65*), were very effective in promoting cholesterol efflux from cholesterol-loaded cell cultures (*38*, *65*). It has been postulated that the apoE-mediated cholesterol efflux may be responsible for the protection of experimental animals from atherosclerosis (*66*). Since apoE is the only apolipoprotein expressed in the brain, it is the logical candidate biomolecule for acting as an acceptor of cellular cholesterol, and thus contributing to the homeostasis of cholesterol and other lipids in the brain. Finally, apoE signaling via lipoprotein receptors may affect neuronal cell functions associated with AD (*67*).

The preceding discussion was intended to draw attention to the need to study different apoE functions in the brain by established in vitro and new in vivo approaches. Crossing of the apoE2-expressing transgenic mice generated in this study with APP-overexpressing transgenic mice is in progress and may provide an answer to the basic question of whether overexpression of apoE2 in the brain is beneficial or detrimental. The use of TF−apoE2 and the PDGF−apoE2 transgenics will also provide information about whether the differences in tissue specific expression of apoE alter the putative protective role of apoE against AD. In addition, crosses of these apoE-expressing transgenic mice with other mouse models with defects in different aspects of lipoprotein metabolism will provide information about how cholesterol homeostatic mechanisms in the circulation and the brain affect the pathogenesis of Alzheimer's disease.

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