Prominent Axonopathy and Disruption of Axonal Transport in Transgenic Mice Expressing Human Apolipoprotein E4 in Neurons of Brain and Spinal Cord

Ina Tesseur,* Jo Van Dorpe,* Koen Bruynseels,*
Francisca Bronfman,* Raf Sciot,[†]
Alfons Van Lommel,[†] and Fred Van Leuven*

From the Experimental Genetics Group,* Center for Human Genetics, Flemish Institute for Biotechnology; and the Department of Pathology,[†] University Hospitals Leuven, K. U. Leuven, Gasthuisberg, Leuven, Belgium

The epsilon 4 allele of the human apolipoprotein E gene (ApoE4) constitutes an important genetic risk factor for Alzheimer's disease. Recent experimental evidence suggests that human ApoE is expressed in neurons, in addition to being synthesized in glial cells. Moreover, brain regions in which neurons express ApoE seem to be most vulnerable to neurofibrillary pathology. The hypothesis that the expression pattern of human ApoE might be important for the pathogenesis of Alzheimer's disease was tested by generating transgenic mice that express human ApoE4 in neurons or in astrocytes of the central nervous system. Transgenic mice expressing human ApoE4 in neurons developed axonal degeneration and gliosis in brain and in spinal cord, resulting in reduced sensorimotor capacities. In these mice, axonal dilations with accumulation of synaptophysin, neurofilaments, mitochondria, and vesicles were documented, suggesting impairment of axonal transport. In contrast, transgenic mice expressing human ApoE4 in astrocytes remained normal throughout life. These results suggest that expression of human ApoE in neurons of the central nervous system could contribute to impaired axonal transport and axonal degeneration. The possible contribution of hyperphosphorylation of protein Tau to the resulting phenotype is discussed. (*Am J Pathol* 2000, 157:1495–1510)

The epsilon 4 allele of the apolipoprotein E (ApoE) gene is associated with Alzheimer's disease (AD), affecting the onset of the disease in an allele dose-dependent manner.^{1,2} ApoE4 is associated with increased plaque load in AD^{3–5} and early onset of neurofibrillary changes.⁶ ApoE4 has also been implicated in poor neurological recovery after head injury, cerebral hemorrhage, and cardiac by-

pass surgery.^{7–12} In addition, ApoE4 was suggested to act as a risk factor for bulbar-onset amyotrophic lateral sclerosis, for Pick's disease, corticobasal degeneration, and progressive supranuclear palsy (characterized by protein Tau-related cytoskeletal pathology), and for inclusion body myositis, although contradictory results have been found.^{13–19}

Epidemiological data do not provide evidence for a direct role of ApoE in central nervous system disorders, and its mechanism of action within the central nervous system is not clear. Originally, it was thought that ApoE present in neurons originated only from endocytosis.^{20–22} Numerous cell culture experiments demonstrated receptor-mediated uptake of ApoE as well as effects on neurite outgrowth and cell-morphology.^{23–29} However, more and more evidence indicates that intraneuronal ApoE might also originate from synthesis by neurons. *In situ* hybridization of brain sections of transgenic mice expressing genomic fragments containing the entire human ApoE locus, including its promoter sequences, revealed expression in neurons, besides astrocytes.^{30–32} Human neuroblastoma cells were shown to synthesize both ApoE mRNA and protein.^{33,34} Moreover, *in situ* hybridization of human brain sections conclusively demonstrated ApoE mRNA in neurons.³⁵ Taken together these results suggest that besides its well-known synthesis by glia cells and its role in lipid transport and metabolism, not only uptake but also synthesis of ApoE by neurons might be important. Interestingly, the brain regions expressing ApoE in neurons seemed most vulnerable to the development of neurofibrillary pathology,³⁶ raising the possibility that the neuronal expression pattern of human ApoE might be important in the pathogenesis of AD.

Supported by the 'Fonds voor Wetenschappelijk Onderzoek' (FWO-Vlaanderen), the Interuniversity-Network for Fundamental Research (IUAP), by the special Action Program for Biotechnology of the Flemish government (VLAB/IWT, COT-008), by the Rooms-fund, by Janssen Research Foundation, and by K. U. Leuven Research and Development.

Accepted for publication July 20, 2000.

Both I. T. and J. V. D. contributed equally to this work.

Address reprint requests to Fred Van Leuven, Ph.D., Dr.Sc., Experimental Genetics Group, Flemish Institute for Biotechnology, Center for Human Genetics, K. U. Leuven, Campus Gasthuisberg O&N 06, B-3000 Leuven, Belgium. E-mail: fredvl@med.kuleuven.ac.be.

To experimentally test this hypothesis, we have generated transgenic mice overexpressing human ApoE4 in neurons or astrocytes of the central nervous system. Initial characterization of these mice showed that the microtubule-associated protein Tau was hyperphosphorylated in the brain of transgenic mice expressing ApoE4 in neurons, but not in mice expressing human ApoE4 in nonneuronal cells.³⁷ Here we present evidence that transgenic mice expressing human ApoE4 in neurons, suffer from axonal degeneration and gliosis in brain and spinal cord. Phenotypically, this resulted in reduced sensorimotor capacities and neurogenic muscle atrophy. Impairment of axonal transport was documented immunohistochemically by accumulation of synaptophysin and ultrastructurally by accumulation of vesicles and mitochondria. In sharp contrast, none of these features were found in mice expressing human ApoE4 in astrocytes.

Experimental Procedures

ApoE4 Transgenic Mice

All transgenic mice contained a 5.5-kb genomic fragment of the human ApoE4 gene, including the four exons and three introns, controlled by the Thy1, platelet-derived growth factor (PDGF), glial fibrillary acidic protein (GFAP), or phosphoglycerate kinase promoters. The transgene was expressed in neurons when under control of the Thy1, PDGF, or phosphoglycerate kinase promoters, and in glial cells only, when under control of the GFAP promoter. Offspring of transgenic mice were genotyped for the presence of the transgene as described.³⁷

Collection of Cerebrospinal Fluid (CSF) and Neuronal and Glial Cell Culture

One μ l of CSF, collected from 3-month-old mice, was used for Western blotting as previously described.³⁸

Primary hippocampal neuronal cultures of 1-day-old Thy1-ApoE4 transgenic and wild-type mice and primary cortical glial cultures of 3-day-old GFAP-ApoE4 transgenic and wild-type mice were prepared as described.³⁹ Hippocampal neurons were grown in the presence of wild-type astrocyte feeder layers for 18 days. Cortical glial cultures of GFAP-ApoE4 transgenic mice were cultured for 11 days, until confluent. Thereafter the medium was removed and the glial cells were grown in serum-free Dulbecco's modified Eagle's medium/F12 medium containing N2 supplement (Life Technologies, Inc., Merelbeke, Belgium) for an additional 72 hours. Medium was collected from both neuron and glial cultures, and 20 μ l of unconcentrated medium was analyzed by Western blotting. Cells were washed twice with phosphate-buffered saline (PBS), scraped from the dishes, and homogenized in 50 μ l (neurons) or 100 μ l (glia) of buffer. Ten μ l of the homogenates were analyzed by Western blotting.

Behavioral Testing

All mice used for behavioral testing were kept under standard light-dark cycle conditions and housed with four mice per cage, having free access to food and water. The open field arena was 1-m diameter with walls 35 cm high, painted black, and lighted diffusely (110 lux). Mice were environmentally adapted to the room 1 hour before testing. Behavioral tests were performed between 13.00 and 17.00. Mice were placed individually on one side of the arena and observed for 10 minutes continuously, but analyzed as two observation blocks of 5 minutes. Total horizontal activity was measured using a computer automated tracking system (PolyTrack, Columbus Instruments, San Diego, CA).

For the forced swim test, mice were allowed to swim freely in a water-tank (105 cm in diameter, 35 cm high), with walls and floor painted white. The tank was filled to a height of 20 cm with opaque water kept at $31 \pm 2^\circ\text{C}$. Total swimming distance was measured with the computerized tracking system and average swimming speed was calculated.

Mice were subjected to a series of sensorimotor tasks to assess muscle strength and equilibrium. The grid suspension test measured the ability of mice to hang upside-down from a wire grid (10 $\times$ 18 cm with 1-mm wires spaced 10 mm apart) for a maximum of 1 minute in two separate trials. The prehensile reflex was measured as the ability of an animal to remain suspended by the forepaws grasped around an elevated horizontal bar (2 mm in diameter) and is considered to be a measure of muscle strength. The traction capacity was scored as the number of hindlimbs that the animal raised to reach the wire. This test is considered to measure equilibrium and muscle tone as well as muscle strength.⁴⁰ Sensorimotor tests were performed as described.⁴⁰

Data were statistically analyzed by one-way analysis of variance and by Student's *t*-test.

All behavioral tests were performed with the genetic status of the mice unknown to the experimenter.

Histology and Immunohistochemistry

Anesthetized mice were perfused with saline followed by 4% paraformaldehyde in PBS. The brains were postfixed for 16 hours in 4% paraformaldehyde in PBS at 4°C and washed in PBS buffer, dehydrated, and embedded in paraffin for sectioning (5 to 7 μm). Alternatively, vibratome sections of 50 μm were cut and transferred to microtiter wells in PBS buffer containing 0.1% sodium azide at 4°C .

Spinal cord was removed and sectioned in four parts (cervical, thoracic, lumbar, and sacral). Muscle biopsies were taken from the quadriceps. Tissues were postfixed overnight in 4% paraformaldehyde in PBS at 4°C , embedded in paraffin, and sectioned (5 to 7 μm).

Paraffin sections were routinely stained with hematoxylin and eosin (H&E) and Bielchowsky's silver impregnation. Immunohistochemical staining of paraffin sections was performed according to standard procedures with

commercially available antibodies to the following antigens: GFAP (1:500; DAKO, Glostrup, Denmark), synaptophysin (1:500, DAKO), ubiquitin (1:200, DAKO), AT8 (Innogenetics, Gent, Belgium), AT180 (Innogenetics), and SMI31 (1:1,000; Affinity, Nottingham, UK). 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 specific antibodies. Secondary antibody was biotinylated goat anti-rabbit or goat anti-mouse IgG followed by StreptABComplex/HRP (DAKO). Final staining was developed with 0.075% 3,3'-diaminobenzidine and 0.01% (w/v) hydrogen peroxide. Sections were mounted on gelatin-coated glass slides, counterstained with hematoxylin, dehydrated, and mounted. Sections were stained with commercially available antibodies to ApoE (1:10,000, DAKO) and NF200 (1:500, Sigma, St Louis, MO).

Hippocampi used for ultrastructural examination, were excised from vibratome sections from mice perfused with 4% paraformaldehyde in PBS and were postfixed in 1.25% glutaraldehyde in PBS. Sciatic nerves were fixed in 1.25% glutaraldehyde in PBS. Semithin and ultrathin sections were prepared in a routine manner after embedding in epon and postfixation with osmium tetroxide.

Quantification of Neurons in the Ventral Horns of the Spinal Cord

Three wild-type FVB mice and four Thy1-ApoE4 transgenic mice that were 12 to 14 months old were transcardially perfused with 4% paraformaldehyde in PBS. Spinal cords were immersion-fixed overnight and embedded in paraffin. Microtome sections (6 μ m) of the cervical and thoracolumbal regions were submitted to standard cresyl-violet staining. Composite images from a 3-charge-coupled device color video camera were collected and assembled with appropriate software (AIS/C 4.0; Imaging Research, St. Catharines, Ontario, Canada) and the number of neurons in the right and left ventral horn in nine series of three successive sections was quantified. Counting of the neurons was performed with the genetic status of the mice unknown to the experimenter.

RNA Extraction and Northern Blotting

Mouse tissues were mechanically homogenized in Trizol Reagent (Life Technologies, Glasgow, UK) and total RNA was isolated as recommended by the manufacturer. Samples of total RNA (10 μ g) were denatured for 10 minutes at 70°C in 50% deionized formamide, 20 mmol/L MOPS, 5 mmol/L sodium acetate, 1 mmol/L ethylenediaminetetraacetic acid, and 2.2 mol/L formaldehyde, pH 8.0, separated by electrophoresis in agarose gels, and the RNA was transferred to Hybond-N membranes (Amersham, Amersham, UK). Membranes were prehybridized for 6 hours at 42°C in 5 $\times$ sodium chloride/sodium

phosphate/ethylenediaminetetraacetic acid, 5 $\times$ Denhardt's, 0.5% sodium dodecyl sulfate, 50% deionized formamide, 100 μ g/ml denatured salmon sperm DNA, and 50 μ g/ml heparin, and subsequently hybridized overnight at 42°C in the same solution supplemented with 10% dextran sulfate and the appropriately [³²P]-radiolabeled DNA probe (2 $\times$ 10⁶ cpm/ml). After washing with 0.3 $\times$ sodium chloride/sodium phosphate/ethylenediaminetetraacetic acid and 0.5% sodium dodecyl sulfate at 68°C for 1 hour, membranes were exposed to Hyperfilm MP (Amersham) for 3 hours to 7 days.

Protein Extraction and Western Blotting

Spinal cord tissue was processed as described for brain tissue,³⁷ and Western blotting was performed after separation of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on precast gels (NOVEX, San Diego, CA). Western blotting was performed with monoclonal antibodies AT8 (Innogenetics), AT180 (Innogenetics), PHF-1 (gift from Dr. P. Davies, Albert Einstein College of Medicine, Bronx, NY), TAU5 (Pharmingen, San Diego, CA), ApoE (DAKO), aGFAP (DAKO), and aSYN (DAKO).

Quantitative analysis of Western blots, assessed by densitometric scanning, was normalized, and three to four mice per transgenic strain were analyzed.

Results

ApoE4 Transgenic Mice

The different transgenic mouse strains were demonstrated to express human ApoE4 in neurons, when under control of the Thy1, PDGF, and PGK gene promoters, and exclusively in astrocytes when under control of the GFAP gene promoter.³⁷

The transgenic human ApoE4 was also expressed in spinal cord, as demonstrated by Northern blotting of total RNA extracts from spinal cord of Thy1-ApoE4 (tae-II), PDGF-ApoE4 (pae-II), GFAP-ApoE4 (gae-I), and PGK-ApoE4 (pgk-I) transgenic mice (Figure 1a). The human ApoE probe used did not react with RNA from wild-type mice or ApoE knockout mice. Western blotting of extracts of spinal cord identified human ApoE4 protein (Figure 1b). Quantification of relative levels of human ApoE4 protein in spinal cord demonstrated that the expression levels in the tae-II and tae-XIII transgenic mice were respectively 3.5- and 12-fold higher than in the pae-II transgenic mice. In the gae-I transgenic mice, ApoE4 expression levels were 6.7-fold higher than in the pae-II transgenic mice.

The cellular distribution of the protein was analyzed immunohistochemically on vibratome sections (Figure 1d). In Thy1-ApoE4 transgenic mice, the ApoE4 protein was present in the neuronal cell bodies in the gray matter (Figure 1, d and C), whereas in GFAP-ApoE4 transgenic mice the ApoE4 protein was found only in astrocytes (Figure 1, d and A), as expected.

Human ApoE4 was demonstrated in CSF and in cell culture by Western blotting. CSF was collected from Thy1-ApoE4, GFAP-ApoE4, and wild-type mice to demonstrate the secretion of human ApoE4 *in vivo* (Figure 1c). Medium of primary hippocampal neurons derived from Thy1-ApoE4 transgenic mice, or primary cortical glial cells derived from GFAP-ApoE4 transgenic mice contained human ApoE4 demonstrating secretion from neurons and from astrocytes (Figure 1, f and g).

Spontaneous Behavior of ApoE4 Transgenic Mice

During the first 2 months of life, all ApoE4 transgenic mice used in this study appeared normal. Thereafter, Thy1-ApoE4 mice manifested loss of body weight, which progressively worsened with aging. When lifted by the tail, Thy1-ApoE4 mice showed an abnormal extension reflex. They retracted both forelimbs and hindlimbs (Figure 1, e and B), whereas nontransgenic mice extended their legs (Figure 1, e and A). During movement in the cage, Thy1-ApoE4 mice showed difficulties with walking and climbing. Transgenic mice of the highest expressing Thy1-ApoE4 strain (tae-XIII) developed the most severe phenotype, and homozygous mice developed the symptoms earlier than their heterozygous littermates, indicating that the symptoms were related to the expression level of the transgene. Thy1-ApoE4 mice died prematurely, which was also more pronounced in the higher expressing strain.³⁷

In PDGF-ApoE4 transgenic mice that were >16 months old, an abnormal extension reflex was observed in a limited number of mice, together with a reduction in body weight. In contrast, abnormal behavior or decreased body weight was never observed in GFAP-ApoE4 transgenic mice, even when >24 months old.

Experimental Testing of Behavior

Spontaneous locomotor activity was measured blindly, in a computerized open field test. Mice used in this test were between 12 and 16 months old. Thy1-ApoE4 transgenic mice were the least active: they traveled only 9.5% of the distance compared to age-matched wild-type mice ($P < 0.001$) (Figure 2E). The PDGF-ApoE4 and GFAP-ApoE4 transgenic mice were normally active in this test (Figure 2E). Neither the computerized analysis nor the visual inspection of the recorded locomotion paths re-

vealed anomalies, such as wall-bumping, circling, or meandering paths, except for one GFAP-ApoE4 mouse, which was excluded from the analysis. This particular mouse was circling around its tail, a phenotype occasionally observed in wild-type mice of the Fvb strain and considered irrelevant to the transgenic phenotype.

In addition to decreased spontaneous locomotion, Thy1-ApoE4 transgenic mice showed a decrease in muscular strength, which was measured by the grid suspension test. At 3 months, the Thy1-ApoE4 transgenic mice already showed a 50% decrease in wire hang time compared to age-matched littermates ($P < 0.005$) (Figure 2G). This reduction further increased with age, as 12- to 16-month-old Thy1-ApoE4 transgenic mice showed a 92% reduction in wire hang time ($P < 0.001$) (Figure 2B). All other 12- to 16-month-old transgenic mice analyzed scored normally in the grid suspension test (Figure 2B).

The decrease in muscle strength in the Thy1-ApoE4 transgenic mice was closely related to the decreased body weight (Figure 2, A and C). At 3 months the body weight of Thy1-ApoE4 transgenic mice was reduced by 7.5% relative to wild-type mice (Figure 2H), although the difference was not significant. At 12 to 16 months however, the body weight of the Thy1-ApoE4 transgenic mice was significantly reduced by 30% ($P < 0.001$) (Figure 2A). In addition, PDGF-ApoE4 transgenic mice of 2 years showed a 20% reduction in body weight ($P < 0.01$), that was not observed in 12- to 16-month-old mice.

When the 12- to 16-month-old mice were subjected to a forced swimming test, no differences in swimming speed were detected between PDGF-ApoE4, GFAP-ApoE4, and wild-type mice. The Thy1-ApoE4 transgenic mice were not tested because preliminary testing revealed that they were not able to swim and would drown during the 2 minutes forced swimming test.

Motor impairment in 3-month-old Thy1-ApoE4 transgenic mice was examined by additional tests. The prehensile reflex, a measure for the muscle strength of the forepaws,⁴⁰ was reduced with 39% ($P < 0.02$) (Figure 2F) in Thy1-ApoE4 transgenic mice compared to wild-type littermates. Traction capacity, a measure for the muscle tone and strength of the hindlimbs,⁴⁰ was reduced by 40% ($P < 0.01$) (Figure 2D) in Thy1-ApoE4 transgenic mice compared to wild-type littermates. These results demonstrate that both forelimbs and hindlimbs were affected. Rod walking, an index for equilibrium and psychomotor integration, was clearly disturbed in Thy1-

Figure 1. Analysis of expression of human ApoE4 mRNA and protein and secretion of human ApoE4 in ApoE4 transgenic mice and abnormal extension reflex in Thy1-ApoE4 transgenic mice. **a:** Northern blot from spinal cord mRNA of representative mice from the four different transgenic ApoE4 strains and wild-type mice. Total spinal cord RNA (10 μ g) was blotted and hybridized for human ApoE4 mRNA and for actin mRNA. **b:** Western blot of proteins extracted from spinal cord of ApoE4 transgenic and wild-type mice. Bands were detected with a polyclonal anti-human-ApoE antibody. **c:** Western blot of human ApoE4 in CSF from wild-type, Thy1-ApoE4 (tae-II), and GFAP-ApoE4 (gae-I) transgenic mice. **d:** Immunohistochemistry for human ApoE4 in spinal cord. Ventral horn of a GFAP-ApoE4 (**A**) transgenic mouse and wild-type (**B**) mouse, and dorsal horn of a Thy1-ApoE4 (**C**) transgenic mouse and wild-type (**D**) mouse. Scale bar, 50 μ m. **e:** Extension reflex in an 8-month-old wild-type (**A**) and an age-matched Thy1-ApoE4 (tae-II) transgenic (**B**) mouse. **f:** Western blotting of human ApoE4 in primary cortical glial cultures derived from three GFAP-ApoE4 (gae-I) transgenic pups. Human ApoE4 was expressed by cultured glial cells (C1, ApoE) and secreted into the medium (M, ApoE). Reaction with the GFAP antibody identified the cells as glial cells (C2, ApoE). **g:** Western blotting of human ApoE4 in primary hippocampal neuronal cultures derived from three Thy1-ApoE4 (tae-XIII) transgenic pups. Human ApoE4 was expressed by transgenic neurons (C1, ApoE) and secreted into the medium (M, ApoE), but was not secreted by wild-type neurons. Reaction with the synaptophysin-antibody identified the cells as neurons (C2, Syn). Brain extract (B, ApoE) of a Thy1-ApoE4 transgenic mouse is shown as reference. Abbreviations: wt, wild type; tae-II, Thy1-ApoE4 line 2; gae-I, GFAP-ApoE4 line 1; pae-II, PDGF-ApoE4 line 2; pgk, PGK-ApoE4 line 1.

Figure 2. Body weight, parameters of muscle strength, and spontaneous locomotion in ApoE4 transgenic and wild-type mice. **A:** Body weight of Thy1-ApoE4 (tae, $n = 6$), GFAP-ApoE4 (gae, $n = 5$), PDGF-ApoE4 (pae, $n = 6$), and PGK-ApoE4 (pgk, $n = 7$) transgenic mice, and wild-type (wt, $n = 8$) mice (12 to 16 months). **B:** Wire hang time for Thy1-ApoE4 (tae, $n = 6$), GFAP-ApoE4 (gae, $n = 5$), PDGF-ApoE4 (pae, $n = 6$), and PGK-ApoE4 (pgk, $n = 7$) transgenic mice and wild-type (wt, $n = 8$) mice (12 to 16 months), as measured in the grid suspension test. **C:** Relation between muscle strength, measured in the grid suspension test, and body weight in individual Thy1-ApoE4 mice (tae Δ , $n = 15$) and wild-type (wt $\blacklozenge$, $n = 17$) littermates. **D:** Traction capacity in Thy1-ApoE4 (tae, $n = 15$) and wild-type (wt, $n = 17$) mice, 3 months old. **E:** Spontaneous locomotor activity, measured as distance traveled in an open field area for 10 minutes, and analyzed in two consecutive rounds of 5 minutes each ($\square = 1'$ to $5'$; $\blacksquare = 5'$ to $10'$). Thy1-ApoE4 (tae, $n = 6$), GFAP-ApoE4 (gae, $n = 5$), PDGF-ApoE4 (pae, $n = 6$), and PGK-ApoE4 (pgk, $n = 7$) transgenic mice and wild-type (wt, $n = 8$) mice, 12 to 16 months old, were used. **F:** Prehensile reflex in Thy1-ApoE4 (tae, $n = 15$) and wild-type (wt, $n = 17$) mice, 3 months old. **G:** Wire hang time for Thy1-ApoE4 (tae, $n = 10$) transgenic mice and wild-type (wt, $n = 13$) mice, 3 months old, measured in the grid suspension test. **H:** Body weight of Thy1-ApoE4 (tae, $n = 10$) transgenic mice and wild-type (wt, $n = 13$) mice of 3 months. All results are expressed as means ($\pm$ SEM).

ApoE4 transgenic mice. Approximately 45% of the 3-month-old Thy1-ApoE4 transgenic mice fell almost immediately from the rod, whereas wild-type mice always completed the task.

Taken together, these results illustrate the sensorimotor defects in mice expressing human ApoE4 in neurons. These defects progressed with age, and in individual mice the decrease in muscle strength was correlated with a decrease in body weight.

Histology and Ultrastructural Examination

Brains and spinal cords of 20 Thy1-ApoE4 transgenic mice, aged 3 to 23 months, were analyzed to determine the neuropathological cause of their phenotype. Routine H&E staining of brain sections showed hypertrophy of astrocytes. Immunostaining for GFAP demonstrated widespread astrogliosis in the hippocampus, neocortex, and amygdala of Thy1-ApoE4 mice, aged 8 months or older (Figure 3A).³⁷ Immunohistochemical staining for ubiquitin revealed small rounded or irregular inclusions in the neuropil of the stratum oriens of the hippocampus³⁷ (Figure 3C) and in the hippocampal fimbria (Figure 3E). Ubiquitin-positive inclusions were also seen in the neocortex and the amygdala, and in the white matter fiber tracts. The inclusions also stained with SMI31, a monoclonal antibody that strongly reacts with a phosphorylated epitope on neurofilament-high, and with NF200, a phosphorylation-independent monoclonal antibody specific for neurofilament-high (Figure 3, H and G). From sections stained for neurofilament it became clear that the inclusions in the neuropil were dilated axons (Figure 3G). In addition, brain sections stained for synaptophysin showed accumulation of synaptophysin in axons in brain white matter fiber tracts, mainly in the hippocampal fimbria, internal and external capsule, corticospinal tract, and corpus callosum (Figure 4, B–D).

The spinal cord gray and white matter of Thy1-ApoE4 mice aged 8 months or older or PDGF-ApoE4 mice aged 20 months or older showed the same pathology as in brain. Ubiquitin and SMI31-positive dilated axons were seen in the neuropil of the gray matter (Figure 5, D and F). White matter fiber tracts displayed dilated axons staining for ubiquitin (Figure 5, H and I) and synaptophysin (Figure 4F). The white matter abnormalities were most obvious in the posterior column (Figure 4F and Figure 5, H and I). Mild gliosis was present in the spinal cord gray and white matter (Figure 5B).

Ultrastructural examination was performed on hippocampi of three Thy1-ApoE4 transgenic mice (13 to 15 months old) and of two age-matched wild-type mice. Transmission electron microscopy demonstrated dilated axons in the stratum oriens and the fimbria, which corresponded to the ubiquitin and synaptophysin-positive inclusions seen by immunohistochemical staining (Figure 7, A and B). The dilation of the axons varied, with the most dilated axons having diameters almost equal to the cell

Figure 3. GFAP staining showing reactive astrogliosis in the hippocampus of an 18-month-old Thy1-ApoE4 mouse (A), but not in an age-matched wild-type mouse (B). Ubiquitin-positive dilated axons (arrows) can be seen in the stratum oriens of the hippocampus (C), hippocampal fimbria (E) and corticospinal tract (J) of Thy1-ApoE4 transgenic mice, but not in wild-type littermates (D, F, and K). Note the fine granularity of the white matter of the hippocampal fimbria and corticospinal tract in the Thy1-ApoE4 transgenic mice (E and J), corresponding to slightly dilated ubiquitin-positive (degenerating) axons. Neurofilament-positive dilated axons (arrows) were observed in the stratum oriens of the hippocampus of Thy1-ApoE4 mice (H), but not in wild-type littermates (I). Detail of a neurofilament-positive dilated axon (G, the nondilated part of the axon is indicated by small arrows). Scale bars, 1 mm (A and B); 50 μ m (C–K and inset in C). CA1, CA2, CA3, regions of hippocampus; DG, dentate gyrus; Py, pyramidal cells of the hippocampus; fi, fimbria of the hippocampus.

Figure 4. Accumulation of synaptophysin in dilated axons of Thy1-ApoE4 transgenic mice. Hippocampal fimbria of wild-type (A) and Thy1-ApoE4 transgenic mice (B) of 18 months. Note the fine granularity in the inclusions (C and D are insets of B), corresponding to vesicular structures. Posterior column of the spinal cord of a wild-type (E) and a PDGF-ApoE4 transgenic (F) mice of 20 months. Scale bars, 50 μ m (A, B, E, and F); 20 μ m (C, D, and inset of F).

body of neurons. Some dilated axons showed degenerative features and were filled with numerous dense and multivesicular bodies and electron dense amorphous material (Figure 7, C and D). Other dilated axons were well-preserved and demonstrated accumulated mitochondria and vesicles (Figure 7, F and G). Dilated axons often had thinned myelin sheaths only 2 to 3 lamellae thick (Figure 7, A and C) and sometimes the myelin sheath was almost completely absent or retracted (Figure 7E). On ultrastructural examination the axonal pathology was more prominent than suggested by the immunohistochemical staining, because many scattered nondilated axons were observed to contain an increased number of mitochondria and vesicles, or dense bodies, which were not present in the age-matched nontransgenic mice (Figure 7A). The perikarya and dendrites present in the ultra-thin sections appeared normal.

Sciatic nerve, of seven Thy1-ApoE4 mice (10 to 18 months old) and four age-matched wild-type mice were examined. Semithin sections revealed damaged axons and scattered macrophages with phagocytosed myelin

(Figure 8; A, D, and F), indicative of Wallerian degeneration. The damaged axons were atrophic and often detached from their myelin sheaths (Figure 8D). Rare axons showed accumulation of electron dense material (Figure 8, B and E). The nerves of the Thy1-ApoE4 transgenic mice showed rare clusters of small axons with thin myelin sheets, indicative of regeneration (Figure 6A).

The functional impairment of the damaged axons was reflected by the skeletal muscle fibers that they innervate. H&E staining of sections from quadriceps skeletal muscles of 8- to 18-month-old ($n = 10$) Thy-1-ApoE4 mice showed muscular atrophy. The muscle atrophy was more advanced in transgenic mice of 18 months than at the age of 12 months. Although 12-month-old Thy1-ApoE4 transgenic mice (tae-II) showed mainly scattered angular atrophic fibers (Figure 6B), 18-month-old Thy1-ApoE4 transgenic mice (tae-II) showed obvious grouping of atrophic fibers, diagnostic for neurogenic muscle atrophy (Figure 6C).

As muscle atrophy was evident in motorically affected Thy1-ApoE4 transgenic mice, neuronal loss in the ventral

Figure 5. GFAP staining showing reactive gliosis in the ventral horn of the spinal cord of an 8-month-old Thy1-ApoE4 mouse (**B**), but not in a wild-type littermate (**A**). SMI31 staining showed neurofilament-positive dilated axons in the dorsal horn of the spinal cord in Thy1-ApoE4 transgenic mice (**D**), but not in wild-type mice (**C**). Ubiquitin-positive dilated axons (**arrows**) in the dorsal horn (**F**) and posterior column of the spinal cord (**H** and **I**) in a 12-month-old Thy1-ApoE4 transgenic mouse but not in a wild-type littermate (**E** and **G**). Scale bars, 50 μ m (**A**, **B**, **G**, and **H**); 20 μ m (**C**–**F**); 50 μ m (**I**). GM, gray matter.

Figure 6. Semithin section of 12-month-old Thy1-ApoE4 transgenic mouse showing a cluster of small axons with a thin myelin sheath, indicative of regeneration (**A**, arrows). H&E staining of the quadriceps skeletal muscle of a sensorimotor impaired Thy1-ApoE4 (tae-II) mouse of 12 months (**B**) and of 18 months (**C**), showing grouping of atrophic (**large arrows**) fibers, which increased with age. Fibers with a normal caliber are indicated by **small arrows**. Scale bars, 25 μ m (**A**); 50 μ m (**B** and **C**).

horn of the spinal cord was examined. No significant reduction in the number of neurons was measured. In wild-type ($n = 3$) and Thy1-ApoE4 transgenic mice ($n = 4$), the average number of neurons was 55.0 ± 4.1 (mean $\pm$ SEM) and 55.2 ± 3.7 (mean $\pm$ SEM) per ventral horn, respectively. Rare pale neurons with loss of Nissl substance were observed in the gray matter of the spinal cord (Figure 9c).

Hyperphosphorylation of Protein Tau in Spinal Cord of Thy1-ApoE4 Transgenic Mice

Western blotting demonstrated hyperphosphorylation of protein Tau in spinal cord of Thy1-ApoE4 transgenic mice. Three monoclonal antibodies (AT8, AT180, and PHF1) directed against known phosphorylated epitopes on the Tau protein, demonstrated increased protein Tau phosphorylation in spinal cord in Thy1-ApoE4 transgenic mice, compared to wild-type littermates (Figure 9a). Immunoblotting with TAU5, a phosphate-independent monoclonal antibody, demonstrated similar protein Tau levels in transgenic and wild-type mice (Figure 9a).

Immunohistochemical staining for protein Tau on spinal cord with the phosphate-dependent monoclonal antibody AT8 revealed strong reactivity in axons and weak somatodendritic staining in the neuronal cell bodies in 12- to 16-month-old wild-type Fvb mice. In 12- to 16-month-old Thy1-ApoE4 transgenic mice, a similar staining pattern was observed, except for some rare neurons with increased AT8 immunoreactivity (Figure 9b, A and C). The phosphate-dependent monoclonal antibody AT180 revealed strong somatodendritic staining, which was not increased, in the Thy1-ApoE4 transgenic mice. These results are in agreement with results obtained by Western blotting and with the increased AT8 immunoreactivity in brain.³⁷ Neither immunohistochemistry nor silver staining revealed neurofibrillary tangles.

Phosphorylation of the 200-kd neurofilament subunit (NF-H) remained unchanged in sensorimotor affected Thy1-ApoE4 transgenic mice, compared to wild-type littermates, as analyzed by antibodies SMI31 and NF200 (results not shown).

Discussion

Expression of human ApoE4 in the brain of transgenic mice was restricted to neurons when under control of the Thy1 and PDGF promoter and to astrocytes, when under control of the GFAP promoter.³⁷ Here, we demonstrated that in the Thy1-ApoE4 transgenic mice, ApoE4 expression was also evident in neurons of the spinal cord, whereas in the GFAP-ApoE4 transgenic mice, ApoE4 expression was restricted to astrocytes.

The present study demonstrated that expression of human ApoE4 in neurons resulted in axonopathy and in disruption of axonal transport, which was accompanied by reduced sensorimotor capacities. The sensorimotor impairment increased with age and neuronal expression level. In contrast, these symptoms were not observed in transgenic mice expressing human ApoE4 in astrocytes.

Figure 7. Ultrastructure of the stratum oriens of the hippocampus and the hippocampal fimbria of 15-month-old Thy1-ApoE4 transgenic mice. **A:** Overview of the stratum oriens of the hippocampus showing degenerative dilated axons (**large arrows**). Normal axons are indicated by **small arrows**. **B:** Higher magnification of a dilated axon containing numerous electron-dense and multivesicular bodies, surrounded by a thinned myelin sheath (**arrows**). **C:** Dilated axon containing electron-dense amorphous material (**arrows**). **D:** Degenerating, only slightly dilated axons (**arrows**) in the hippocampal fimbria, filled with degenerative organelles and amorphous material. **E:** Hippocampal fimbria with grossly dilated degenerating axon, surrounded by nonaffected axons. The myelin sheath is thin or absent. Scale bar, 1 μ m. Scale bar in **B** also applies to **C**. Accumulation of mitochondria in dilated, nondegenerative axons in 15-month-old Thy1-ApoE4 transgenic mice (**F** and **G**). Note the accumulation of small vesicles (**arrows**) in **F**. Scale bar, 2 μ m (**F** and **G**).

Figure 8. Ultrastructure of sciatic nerve of 15-month-old Thy1-ApoE4 transgenic mouse. **A:** Semithin section showing macrophages (arrows) filled with myelin debris, indicative of Wallerian degeneration. **B:** Semithin section of a collapsed axon surrounded by a thinned and partially disrupted myelin sheath (large arrow); the smaller arrows indicate macrophages. **C:** Affected axon with accumulation of mitochondria and amorphous material. **D:** Higher magnification of an axon partially filled with granular and electron-dense amorphous material (arrows). **E:** High magnification of an atrophic collapsed axon. **F:** A macrophage (arrows) filled with myelin debris. Scale bars, 10 μm (A, B, and E); 1 μm (C, D, and F).

Examination of brain and spinal cord revealed axonal dilations staining for ubiquitin and neurofilament in the neuropil of the gray matter and in white matter tracts of affected ApoE4 transgenic mice. Moreover, in numerous axons we observed accumulation of synaptophysin, a protein associated with precursors of synaptic vesicles and transported by fast axonal transport. In addition, ultrastructural analysis revealed accumulation of mitochondria and vesicles in dilated axons. Astrogliosis, a general indicator of CNS injury, was observed in neocortex, hippocampus, amygdala, and in spinal cord. This is not surprising because we observed widespread axonopathy. Ultrathin sections revealed degenerated axons

corresponding to the immunohistochemically observed accumulation of ubiquitinated proteins. Taken together, these results suggest that the neuronal expression of human ApoE4 contributed to a disruption of axonal transport and to axon degeneration. Surprisingly, no neuronal loss was established, although some neurons with loss of Nissl substance and increased AT8 immunoreactivity were observed. In this context, the observed impairment of axonal transport in AD neurons, their loss of polarity and subsequent degeneration,^{41,42} and an increased vulnerability to neuronal death in those brain regions that express ApoE in neurons,^{35,36} is of special interest. Although great care should be taken in extrapolating re-

Figure 9. **a:** Hyperphosphorylation of protein Tau in spinal cord of 18-month-old Thy1-ApoE4 (tae-XIII) transgenic mice, compared to wild-type (wt) littermates. Four independent Tau-specific monoclonal antibodies (AT8, PHF1, AT180, and TAU5) were used to detect bands. Western blots shown are representative examples. **b:** Immunohistochemical staining with the phosphorylation-dependent monoclonal antibody AT8, demonstrating rare neurons with strong somatodendritic staining (arrows) in the spinal cord of 12-month-old Thy1-ApoE4 (tae-II) transgenic mice (A and C, arrows). Neurons with strong somatodendritic staining with AT8 were not present in wild-type littermates (B and D). Scale bars, 50 μm (A–D). WM, white matter. **c:** Cresyl violet staining of the ventral horn of a 12-month-old Thy1-ApoE4 (tae-II) transgenic mouse showing loss of Nissl substance in some neurons (arrows). Scale bar, 50 μm.

sults from mouse models to humans, it is possible to speculate that the axonal degeneration we observe in our mice represents some form of neuronal degeneration.

Tau proteins are microtubule-associated proteins that help to regulate the dynamics of the microtubular network, for which the degree of their phosphorylation is important.⁴³ In AD, Tau is abnormally hyperphosphory-

lated and aggregates into paired helical filaments.⁴⁴ Furthermore, Tau in AD is dislocated mainly to the somatodendritic compartment of neurons,⁴⁵ whereas protein Tau concentration was reported to be elevated in AD brain.⁴⁶ Overexpression of protein Tau inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmic reticulum in neuro-2a cells,⁴⁷ and causes

prominent axonopathy in htau-40 transgenic mice.⁴⁸ These data strengthen the importance of proper control by posttranslational modification of the microtubule-associated protein Tau, its localization, and intracellular concentration, *in vivo*. We have shown that expression of ApoE4 in neurons results in hyperphosphorylation of protein Tau in brain³⁷ and in spinal cord (this study). As the Tau protein is considered to be a major component of the short cross-bridges between microtubules,⁴⁹ and as *in vitro* data have demonstrated that hyperphosphorylation of protein Tau reduced its affinity for microtubules,^{50,51} hyperphosphorylation of protein Tau is likely to influence cross-bridge formation or stability of microtubules and thereby affect normal axonal transport. In the Thy1-ApoE4 transgenic mice it is possible that because of increased protein Tau phosphorylation, the dynamics of the cytoskeleton are impaired, which could contribute to disruption of the axonal transport and result in the degeneration of axons. In this context, the so-called dying back of axons, beginning from the most distal regions, which would lead to the degeneration of neurons, is of special interest.^{45,52}

At this moment the mechanism whereby expression of human ApoE4 in neurons is leading to protein Tau hyperphosphorylation is not known. Recently, it was shown that extracellular reelin binds to the very low density lipoprotein receptor (VLDLR) and ApoER2 lipoprotein receptors, thereby inducing tyrosine phosphorylation of Disabled-1, which activates tyrosine kinases. The binding was competed for by ApoE-lipoproteins. In addition, mice lacking both VLDLR and ApoER2 showed increased protein Tau phosphorylation, as did reelin knockout mice.^{53,54} One can speculate that co-expression of human ApoE4 with ApoER2 and VLDLR in neurons could result in aberrant triggering of intracellular signal transduction pathways leading to protein Tau phosphorylation. On the other hand, ApoE particles secreted by neurons might differ from those secreted by astrocytes, which might result in a differential interaction with ApoE-lipoprotein receptors such as VLDLR and ApoER2. Obviously, this hypothesis does not exclude that other mechanisms might be involved.

Recently it was shown that ApoE normally resides within the secretory pathway and that direct expression of ApoE in the cytoplasm of neuro-2a cells is toxic.⁵⁵ The human ApoE4 protein in our transgenic mice is not expected to escape from the secretory pathway. Although we cannot exclude leakage into the cytosol, and hence a direct cytotoxic effect, this is not considered likely. Indeed, the transgene contains the signal sequence of ApoE, which directs it to the endoplasmic reticulum, and the ApoE4 protein is secreted from neurons derived from Thy1-ApoE4 transgenic mice and from astrocytes derived from GFAP-ApoE4 transgenic mice. In addition, human ApoE4 was found in the CSF of both GFAP-ApoE4 and Thy1-ApoE4 transgenic mice.

Synthetic peptides, as well as a 22-kd thrombin-cleavage fragment derived from ApoE, have been demonstrated to exhibit cytotoxicity^{56–59} and cause neurite degeneration.⁶⁰ This process was suggested to be receptor-mediated.⁵⁸ We did not observe 22-kd ApoE fragments in

the ApoE4 transgenic mice with the method that we used, which argues against a role for these fragments in the pathology of the Thy1-ApoE4 transgenic mice.

Neurodegeneration of the central nervous system and peripheral sensory nerve defects have been reported in mice lacking ApoE.^{61,62} Because endogenous mouse ApoE levels remained unchanged in Thy1-ApoE4 transgenic mice, the observed axon degeneration in the brain and in spinal cord and Wallerian degeneration in sciatic nerve, cannot be attributed to a down-regulation or absence of endogenous ApoE.

Other transgenic mice, generated in our laboratory, that express high levels of unrelated proteins, under the control of the mouse Thy1 gene promoter, did not show axonopathy and remained healthy throughout life. Therefore we exclude the possibility of a general neurotoxic effect by overexpressing high levels of any protein in the central nervous system, under control of the mouse Thy1 gene promoter. In addition, PDGF-ApoE4 transgenic mice, expressing human ApoE4 in neurons at a lower level, also showed signs of axonopathy, at age 2 years or older. The GFAP-ApoE4 transgenic mice on the other hand convincingly demonstrate that even very high levels of human ApoE4 per se, are not toxic or detrimental for normal functioning of the mouse brain.

Other transgenic mice, expressing human ApoE in neurons have been generated.^{30–32,61–63} Effects on learning and exploratory behavior were demonstrated in female NSE-ApoE4 transgenic mice at the age of 6 months, but no axonopathy was reported.⁶³ In the PDGF-ApoE4 transgenic mice, we observed a similar, but less severe phenotype as in the Thy1-ApoE4 transgenic mice, but only when they aged to ~2 years. It would be interesting to investigate whether axonopathy and motor impairments are also present in old NSE-ApoE⁶³ and human ApoE mice.^{30–32}

In conclusion, we demonstrate that expression of human ApoE4 in neurons, *in vivo*, resulted in axonopathy and impairment of axonal transport, as opposed to expression in nonneuronal cells. We further demonstrated that this is not because of a general toxic effect of human ApoE4 in mouse brain. The current mouse model offers an excellent opportunity to investigate the mechanism by which ApoE could be involved in protein Tau phosphorylation and in neurodegeneration.

Acknowledgments

We thank Dr. P. Davies for donating PHF1 antibody; C. Armée, T. Boon, M. Gillis, D. Kirali, and R. Renwart for technical assistance; and C. Vochten for help with administration.

References

1. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA: Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families [see comments]. *Science* 1993; 261:921–923
2. Strittmatter WJ, Saunders AM, Goedert M, Weisgraber KH, Dong LM,

- Jakes R, Huang DY, Pericak-Vance M, Schmechel D, Roses AD: Isoform-specific interactions of apolipoprotein E with microtubule-associated protein tau: implications for Alzheimer disease. *Proc Natl Acad Sci USA* 1994, 91:11183-11186
3. Olichney JM, Hansen LA, Galasko D, Saitoh T, Hofstetter CR, Katzman R, Thal LJ: The apolipoprotein E epsilon 4 allele is associated with increased neuritic plaques and cerebral amyloid angiopathy in Alzheimer's disease and Lewy body variant. *Neurology* 1996, 47:190-196
4. Nagy Z, Esiri MM, Jobst KA, Johnston C, Litchfield S, Sim E, Smith AD: Influence of the apolipoprotein E genotype on amyloid deposition and neurofibrillary tangle formation in Alzheimer's disease. *Neuroscience* 1995, 69:757-761
5. Gearing M, Mori H, Mirra SS: A beta-peptide length and apolipoprotein E genotype in Alzheimer's disease. *Ann Neurol* 1996, 39:395-399
6. Ghebremedhin E, Schultz C, Braak E, Braak H: High frequency of apolipoprotein E epsilon4 allele in young individuals with very mild Alzheimer's disease-related neurofibrillary changes. *Exp Neurol* 1998, 153:152-155
7. Alberts MJ, Graffagnino C, McClenny C, DeLong D, Strittmatter WJ, Saunders AM, Roses AD: ApoE genotype and survival from intracerebral haemorrhage [letter]. *Lancet* 1995, 346:575
8. Jordan BD, Relkin NR, Ravdin LD, Jacobs AR, Bennett A, Gandy S: Apolipoprotein E epsilon4 associated with chronic traumatic brain injury in boxing [see comments]. *JAMA* 1997, 278:136-140
9. Mayeux R, Ottman R, Maestre G, Ngai C, Tang MX, Ginsberg H, Chun M, Tycko B, Shelanski M: Synergistic effects of traumatic head injury and apolipoprotein-epsilon 4 in patients with Alzheimer's disease [see comments]. *Neurology* 1995, 45:555-557
10. Nicoll JA, Roberts GW, Graham DI: Apolipoprotein E epsilon 4 allele is associated with deposition of amyloid beta-protein following head injury [see comments]. *Nat Med* 1995, 1:135-137
11. Reed T, Carmelli D, Swan GE, Breitner JC, Welsh KA, Jarvik GP, Deeb S, Auwerx J: Lower cognitive performance in normal older adult male twins carrying the apolipoprotein E epsilon 4 allele. *Arch Neurol* 1994, 51:1189-1192
12. Tardiff BE, Newman MF, Saunders AM, Strittmatter WJ, Blumenthal JA, White WD, Croughwell ND, Davis Jr RD, Roses AD, Reves JG: Preliminary report of a genetic basis for cognitive decline after cardiac operations. The Neurologic Outcome Research Group of the Duke Heart Center. *Ann Thorac Surg* 1997, 64:715-720
13. al-Chalabi A, Enayat ZE, Bakker MC, Sham PC, Ball DM, Shaw CE, Lloyd CM, Powell JF, Leigh PN: Association of apolipoprotein E epsilon 4 allele with bulbar-onset motor neuron disease. *Lancet* 1996, 347:159-160
14. Askanas V, Engel WK, Mirabella M, Weisgraber KH, Saunders AM, Roses AD, McFerrin J: Apolipoprotein E alleles in sporadic inclusion-body myositis and hereditary inclusion-body myopathy [letter]. *Ann Neurol* 1996, 40:264-265
15. Bachus R, Bader S, Gessner R, Ludolph AC: Lack of association of apolipoprotein E epsilon 4 allele with bulbar-onset motor neuron disease [letter]. *Ann Neurol* 1997, 41:417
16. Moulard B, Sefiani A, Laamri A, Malafosse A, Camu W: Apolipoprotein E genotyping in sporadic amyotrophic lateral sclerosis: evidence for a major influence on the clinical presentation and prognosis. *J Neurol Sci* 1996, 139(suppl):34-37
17. Smith RG, Haverkamp LJ, Case S, Appel V, Appel SH: Apolipoprotein E epsilon 4 in bulbar-onset motor neuron disease [letter]. *Lancet* 1996, 348:334-335
18. Schneider JA, Gearing M, Robbins RS, de l'Aune W, Mirra SS: Apolipoprotein E genotype in diverse neurodegenerative disorders. *Ann Neurol* 1995, 38:131-135
19. Love S, Nicoll JA, Lowe J, Sherriff F: Apolipoprotein E allele frequencies in sporadic inclusion body myositis. *Muscle Nerve* 1996, 19:1605-1607
20. Han SH, Hulette C, Saunders AM, Einstein G, Pericak-Vance M, Strittmatter WJ, Roses AD, Schmechel DE: Apolipoprotein E is present in hippocampal neurons without neurofibrillary tangles in Alzheimer's disease and in age-matched controls. *Exp Neurol* 1994, 128:13-26
21. Han SH, Einstein G, Weisgraber KH, Strittmatter WJ, Saunders AM, Pericak-Vance M, Roses AD, Schmechel DE: Apolipoprotein E is localized to the cytoplasm of human cortical neurons: a light and electron microscopic study. *J Neuropathol Exp Neurol* 1994, 53:535-544
22. Metzger RE, LaDu MJ, Pan JB, Getz GS, Frail DE, Falduto MT: Neurons of the human frontal cortex display apolipoprotein E immunoreactivity: implications for Alzheimer's disease. *J Neuropathol Exp Neurol* 1996, 55:372-380
23. DeMattos RB, Curtiss LK, Williams DL: A minimally lipidated form of cell-derived apolipoprotein E exhibits isoform-specific stimulation of neurite outgrowth in the absence of exogenous lipids or lipoproteins. *J Biol Chem* 1998, 273:4206-4212
24. Holtzman DM, Pitas RE, Kilbridge J, Nathan B, Mahley RW, Bu G, Schwartz AL: Low density lipoprotein receptor-related protein mediates apolipoprotein E-dependent neurite outgrowth in a central nervous system-derived neuronal cell line. *Proc Natl Acad Sci USA* 1995, 92:9480-9484
25. Nathan BP, Bellosta S, Sanan DA, Weisgraber KH, Mahley RW, Pitas RE: Differential effects of apolipoproteins E3 and E4 on neuronal growth in vitro. *Science* 1994, 264:850-852
26. Nathan BP, Chang KC, Bellosta S, Brisch E, Ge N, Mahley RW, Pitas RE: The inhibitory effect of apolipoprotein E4 on neurite outgrowth is associated with microtubule depolymerization. *J Biol Chem* 1995, 270:19791-19799
27. Bellosta S, Nathan BP, Orth M, Dong LM, Mahley RW, Pitas RE: Stable expression and secretion of apolipoproteins E3 and E4 in mouse neuroblastoma cells produces differential effects on neurite outgrowth. *J Biol Chem* 1995, 270:27063-27071
28. Fagan AM, Bu G, Sun Y, Daugherty A, Holtzman DM: Apolipoprotein E-containing high density lipoprotein promotes neurite outgrowth and is a ligand for the low density lipoprotein receptor-related protein. *J Biol Chem* 1996, 271:30121-30125
29. Puttfarcken PS, Manelli AM, Falduto MT, Getz GS, LaDu MJ: Effect of apolipoprotein E on neurite outgrowth and beta-amyloid-induced toxicity in developing rat primary hippocampal cultures. *J Neurochem* 1997, 68:760-769
30. Xu PT, Schmechel D, Rothrock-Christian T, Burkhardt DS, Qiu HL, Popko B, Sullivan P, Maeda N, Saunders AM, Roses AD, Gilbert JR: Human apolipoprotein E2, E3, and E4 isoform-specific transgenic mice: human-like pattern of glial and neuronal immunoreactivity in central nervous system not observed in wild-type mice. *Neurobiol Dis* 1996, 3:229-245
31. Xu PT, Gilbert JR, Qiu HL, Rothrock-Christian T, Settles DL, Roses AD, Schmechel DE: Regionally specific neuronal expression of human APOE gene in transgenic mice. *Neurosci Lett* 1998, 246:65-68
32. Roses AD, Gilbert J, Xu PT, Sullivan P, Popko B, Burkhardt DS, Christian-Rothrock T, Saunders AM, Maeda N, Schmechel DE: Cis-acting human ApoE tissue expression element is associated with human pattern of intraneuronal ApoE in transgenic mice. *Neurobiol Aging* 1998, 19:S53-S58
33. Soulié Cathia VM, Laetitia Dupont-Wallois, Marie-Christine Chartier-Harlin, Jean-Claude Beauvillain, André Delacourte, Marie-Laure Cailliet-Boudin: Synthesis of apolipoprotein E (ApoE) mRNA by human neuronal-type SK NSH-SY 5Y cells and its regulation by nerve growth factor and ApoE. *Neurosci Lett* 1999, 265:147-150
34. Dupont-Wallois L, Soulie C, Sergeant N, Wavrant-de Wrieze N, Chartier-Harlin MC, Delacourte A, Cailliet-Boudin ML: ApoE synthesis in human neuroblastoma cells. *Neurobiol Dis* 1997, 4:356-364
35. Xu PT, Gilbert JR, Qiu HL, Ervin J, Rothrock-Christian TR, Hulette C, Schmechel DE: Specific regional transcription of apolipoprotein E in human brain neurons [In Process Citation]. *Am J Pathol* 1999, 154:601-611
36. Einstein G, Patel V, Bautista P, Kenna M, Melone L, Fader R, Karson K, Mann S, Saunders AM, Hulette C, Mash D, Roses AD, Schmechel DE: Intraneuronal ApoE in human visual cortical areas reflects the staging of Alzheimer disease pathology. *J Neuropathol Exp Neurol* 1998, 57:1190-1201
37. Tesseur I, Van Dorpe J, Spittaels K, Van Den Haute C, Moechars D, Van Leuven F: Expression of human apolipoprotein E4 in neurons causes hyperphosphorylation of protein Tau in the brains of transgenic mice. *Am J Pathol* 2000, 156:951-964
38. Van Dorpe J, Smeijers L, Dewachter I, Nuyens D, Spittaels K, Van den Haute C, Mercken M, Moechars D, Laenen I, Kuiperi C, Brynseels K, Tesseur I, Loos R, Vanderstichele H, Checler F, Sciot R, Van Leuven F: Prominent cerebral amyloid angiopathy in transgenic mice over-

- expressing the London mutant of human APP in neurons. *Am J Pathol* 2000, 157:1283–1298
39. Annaert WG, Levesque L, Craessaerts K, Dierinck I, Snellings G, Westaway D, George-Hyslop PS, Cordell B, Fraser P, De Strooper B: Presenilin 1 controls gamma-secretase processing of amyloid precursor protein in pre-golgi compartments of hippocampal neurons. *J Cell Biol* 1999, 147:277–294
40. Lamberty Y, Gower AJ: Age-related changes in spontaneous behavior and learning in NMRI mice from middle to old age. *Physiol Behav* 1992, 51:81–88
41. Dessi F, Colle MA, Hauw JJ, Duyckaerts C: Accumulation of SNAP-25 immunoreactive material in axons of Alzheimer's disease. *Neuroreport* 1997, 8:3685–3689
42. Nakamura Y, Takeda M, Yoshimi K, Hattori H, Hariguchi S, Kitajima S, Hashimoto S, Nishimura T: Involvement of clathrin light chains in the pathology of Alzheimer's disease. *Acta Neuropathol (Berl)* 1994, 87:23–31
43. Delacourte A, Buee L: Normal and pathological Tau proteins as factors for microtubule assembly. *Int Rev Cytol* 1997, 171:167–224
44. Wischik CM, Novak M, Edwards PC, Klug A, Tichelaar W, Crowther RA: Structural characterization of the core of the paired helical filament of Alzheimer disease. *Proc Natl Acad Sci USA* 1988, 85:4884–4888
45. Braak E, Braak H, Mandelkow EM: A sequence of cytoskeleton changes related to the formation of neurofibrillary tangles and neuropil threads. *Acta Neuropathol (Berl)* 1994, 87:554–567
46. Khatoon S, Grundke-Iqbal I, Iqbal K: Brain levels of microtubule-associated protein tau are elevated in Alzheimer's disease: a radio-immuno-slot-blot assay for nanograms of the protein. *J Neurochem* 1992, 59:750–753
47. Ebner A, Godemann R, Stamer K, Illenberger S, Trinczek B, Mandelkow E: Overexpression of tau protein inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmic reticulum: implications for Alzheimer's disease. *J Cell Biol* 1998, 143:777–794
48. Spittaels K, Van den Haute C, Van Dorpe J, Bruynseels K, Vandezande K, Laenen I, Geerts H, Mercken M, Sciot R, Van Lommel A, Loos R, Van Leuven F: Prominent axonopathy in the brain and spinal cord of transgenic mice overexpressing four-repeat human tau protein. *Am J Pathol* 1999, 155:2153–2165
49. Harada A, Oguchi K, Okabe S, Kuno J, Terada S, Ohshima T, Sato-Yoshitake R, Takei Y, Noda T, Hirokawa N: Altered microtubule organization in small-calibre axons of mice lacking tau protein. *Nature* 1994, 369:488–491
50. Bramblett GT, Goedert M, Jakes R, Merrick SE, Trojanowski JQ, Lee VM: Abnormal tau phosphorylation at Ser396 in Alzheimer's disease recapitulates development and contributes to reduced microtubule binding. *Neuron* 1993, 10:1089–1099
51. Biernat J, Gustke N, Drewes G, Mandelkow EM, Mandelkow E: Phosphorylation of Ser262 strongly reduces binding of tau to microtubules: distinction between PHF-like immunoreactivity and microtubule binding. *Neuron* 1993, 11:153–163
52. Trojanowski JQ, Lee VM: Phosphorylation of paired helical filament tau in Alzheimer's disease neurofibrillary lesions: focusing on phosphatases. *FASEB J* 1995, 9:1570–1576
53. Hiesberger T, Trommsdorff M, Howell BW, Goffinet A, Mumby MC, Cooper JA, Herz J: Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. *Neuron* 1999, 24:481–489
54. Trommsdorff M, Gotthardt M, Hiesberger T, Shelton J, Stockinger W, Nimpf J, Hammer RE, Richardson JA, Herz J: Reeler/disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. *Cell* 1999, 97:689–701
55. DeMattos RB, Thorngate FE, Williams DL: A test of the cytosolic apolipoprotein E hypothesis fails to detect the escape of apolipoprotein E from the endocytic pathway into the cytosol and shows that direct expression of apolipoprotein E in the cytosol is cytotoxic. *J Neurosci* 1999, 19:2464–2473
56. Clay MA, Anantharamaiah GM, Mistry MJ, Balasubramaniam A, Harmony JA: Localization of a domain in apolipoprotein E with both cytostatic and cytotoxic activity. *Biochemistry* 1995, 34:11142–11151
57. Marques MA, Tolar M, Harmony JA, Crutcher KA: A thrombin cleavage fragment of apolipoprotein E exhibits isoform-specific neurotoxicity. *Neuroreport* 1996, 7:2529–2532
58. Tolar M, Marques MA, Harmony JAK, Crutcher KA: Neurotoxicity of the 22 kDa thrombin-cleavage fragment of apolipoprotein E and related synthetic peptides is receptor-mediated. *J Neurosci* 1997, 17:5678–5686
59. Moulder KL, Narita M, Chang LK, Bu G, Johnson Jr EM: Analysis of a novel mechanism of neuronal toxicity produced by an apolipoprotein E-derived peptide. *J Neurochem* 1999, 72:1069–1080
60. Crutcher KA, Clay MA, Scott SA, Tian X, Tolar M, Harmony JA: Neurite degeneration elicited by apolipoprotein E peptides. *Exp Neurol* 1994, 130:120–126
61. Masliah E, Mallory M, Ge N, Alford M, Veinbergs I, Roses AD: Neurodegeneration in the central nervous system of apoE-deficient mice. *Exp Neurol* 1995, 136:107–122
62. Fullerton SM, Strittmatter WJ, Matthew WD: Peripheral sensory nerve defects in apolipoprotein E knockout mice. *Exp Neurol* 1998, 153:156–163
63. Raber J, Wong D, Buttini M, Orth M, Bellosta S, Pitas RE, Mahley RW, Mucke L: Isoform-specific effects of human apolipoprotein E on brain function revealed in ApoE knockout mice: increased susceptibility of females. *Proc Natl Acad Sci USA* 1998, 95:10914–10919