Apolipoprotein E allelic influence on human cerebrospinal fluid apolipoproteins

Kathleen S. Montine,* Casey N. Bassett,* Joyce J. Ou,* William R. Markesbery,§ Larry L. Swift,* and Thomas J. Montine[1,*,†]

Departments of Pathology,* and Pharmacology,† and Center for Molecular Neurosciences,† Vanderbilt University Medical Center, Nashville, TN 37232, and Departments of Pathology, Neurology, and the Sanders-Brown Center on Aging,§ University of Kentucky Medical Center, Lexington, KY 40536

Abstract **The major apolipoproteins (apo) on human cerebrospinal fluid lipoproteins are apoA-I and apoE. Given the association between inheritance of the ε4 allele of the apoE gene (APOE4) and increased susceptibility to Alzheimer's disease, we tested the hypothesis that cerebrospinal fluid apolipoproteins may be influenced by APOE genotype and Alzheimer's disease. Lipoprotein fractions (d < 1.210 g/ml) were isolated from cerebrospinal fluid obtained from individuals with different APOE genotypes and with or without pathologically verified Alzheimer's disease. Apolipoproteins were separated by SDS-polyacrylamide gel electrophoresis and identified by silver nitrate staining, Western blotting, and N-terminal amino acid sequencing. Four protein species were detected by silver nitrate staining in subjects with an APOE3 allele: apoA-I, apoE monomer, apoE-apoA-II heterodimer, and apoE homodimer. In APOE4 homozygotes, only apoA-I and apoE monomer were detected. ApoA-II homodimer was demonstrated in all subjects by Western blotting. The relative levels of apoE- and apoA-II-containing apolipoproteins correlated with APOE genotype but were not altered by Alzheimer's disease. In contrast to apoE, no apoA-II immunoreactivity was observed with pathological structures in Alzheimer's disease brain. These differences in cerebrospinal fluid apolipoproteins may influence lipoprotein trafficking and may be an element in the stratification of risk for Alzheimer's disease with APOE genotype.—**Montine, K. S., C. N. Bassett, J. J. Ou, W. R. Markesbery, L. L. Swift, and T. J. Montine. **Apolipoprotein E allelic influence on human cerebrospinal fluid apolipoproteins.** ***J. Lipid Res.*** **1998.** 39: **2443–2451.**

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The presence of lipoproteins in human cerebrospinal fluid (CSF) was first reported by Swahn, Bronnestam, and Dencker (1) in 1961. Roheim and coworkers (2) subsequently identified specific apolipoproteins in human CSF and demonstrated that these were associated with the d < 1.210 g/ml fraction. Further studies characterized CSF lipoproteins and showed that there are two major particles with sizes in the range of plasma high density lipoprotein (HDL) and low density lipoprotein (LDL) (3, 4). The two most abundant CSF apolipoproteins are apoA-I and apoE; CSF lipoproteins do not contain apoB. ApoE in the human CSF is derived from astrocytes in brain and not from plasma (1, 5–7). In contrast, apoA-I in human CSF is not produced in brain, but is derived from plasma (3, 5). Although the mechanisms by which apoA-I crosses the blood–brain barrier are not known, it has been proposed that apoA-I-enriched HDL particles are transported paracellularly between cerebral endothelial cells (8). If so, apoA-II might also be expected in CSF because apoA-II is the other major apolipoprotein of plasma HDL. To date, there has been no definitive identification of apoA-II in human CSF. Although Roheim and coworkers (2) initially presented immunochemical evidence for apoA-II in human CSF, a result recently corroborated (9), other laboratories have published conflicting results about apoA-II in human CSF lipoproteins (3, 4).

Little is known about the role of apoE or the functional differences among apoE isoforms in central nervous system (CNS) lipoprotein metabolism. Humans have three common alleles of the apolipoprotein E gene (APOE): ε2 allele (APOE2), ε3 allele (APOE3), and ε4 allele (APOE4) (10, 11). The most common allele in humans is APOE3, followed by APOE4 and APOE2, with the corresponding isoforms being apoE3, apoE4, and apoE2, respectively. ApoE4 and apoE2 differ from apoE3 by single amino acid substitutions: $cys^{112} \rightarrow$ arg in apoE4 and $arg^{158} \rightarrow$ cys in apoE2. Thus apoE2 has two cysteines, apoE3 has one cysteine, and apoE4 is devoid of cysteines. These differences in primary structure underlie the differences among apoE isoforms with respect to disulfide bond formation, low density lipoprotein (LDL) receptor binding, and plasma

Abbreviations: AD, Alzheimer's disease; CSF, cerebrospinal fluid; apo, apolipoprotein; APOE, human apolipoprotein E gene; DTNB, 5,5′-dithiobis(2-nitrobenzoate); HDL, high density lipoprotein; LDL, low density lipoprotein.

[1]To whom correspondence should be addressed.

lipoprotein distribution. Furthermore, in plasma, the differences in apoE isoforms are known to alter lipoprotein metabolism and to contribute to human diseases, such as hyperlipoproteinemia and atherosclerosis.

In addition to influencing plasma lipoprotein metabolism and altering the risk for vascular disease, inheritance of different APOE alleles appears to modify the pathogenesis of Alzheimer's disease (AD) in some populations (12). Specifically, inheritance of APOE4 is associated with an increased risk of developing AD and decreased average age of onset (13). Moreover, APOE4 has been associated with poorer neurological outcome in patients afflicted with cerebral hemorrhage and closed head injury or undergoing cardiopulmonary bypass surgery, suggesting a broader role for apoE isoforms in the pathogenesis of degenerative and destructive lesions of the CNS (14–16). Although several mechanisms have been proposed, it is not yet known which apoE isoform-specific effects underlie the increased risk for AD.

The seminal work on CSF lipoproteins was performed before there was a clear understanding of the different human APOE alleles; therefore, the influence of different APOE alleles on human CSF apolipoproteins was not addressed. Moreover, there have been conflicting reports on whether significant differences in CSF apoE concentrations exist between AD patients and control subjects (17–19). Previously, we examined the lipid constituents of CSF lipoproteins and showed significant differences between patients with AD and age-matched control subjects that were not related to inheritance of APOE3 or APOE4 (20). In the present study we have examined the apolipoproteins of CSF lipoproteins and have tested the hypothesis that the molecular distribution of CSF apolipoproteins is influenced by APOE alleles and also may be affected by AD.

METHODS

Rabbit polyclonal anti-apoE antiserum was obtained from DAKO (Carpinteria, CA). Goat polyclonal anti-apoA-II, goat anti-rabbit alkaline phosphatase-conjugated IgG, rabbit anti-goat alkaline phosphatase-conjugated IgG, rabbit polyclonal anti-human apoA-I antiserum, and recombinant human apoA-II were purchased from Calbiochem (San Diego, CA). Gels for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), Immunlite and polyvinylidene difluoride (PVDF) membranes, and blotting grade Tween were bought from Bio-Rad (Hercules, CA). Chemiluminescence substrate was obtained from Schleicher and Schuell (Keene, NH). Photographic film was from Eastman Kodak Co. (Rochester, NY). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Sixteen cases were selected from autopsies performed in 1995 or 1996 at Vanderbilt University Medical Center and the University of Kentucky Medical Center. An additional two AD cases were obtained in 1998**.** All AD patients carried a clinical diagnosis of "probable AD" during life and were verified as AD postmortem using standardized criteria. Control subjects were age-matched individuals without clinical evidence of dementia or other neurological disease and who had annual neuropsychological testing with all test scores in the normal range. All control subjects also underwent postmortem examination and were demonstrated to be free of significant neuropathological changes. No case with a postmortem interval greater than 5 h was included. After informed consent, CSF was removed postmortem from the lateral ventricles, sedimented at 1000 *g* for 10 min, and frozen at −80°C until analyzed. After aspiration of CSF, brains were removed and weighed prior to fixation and subsequent histopathological examination. APOE genotype was determined postmortem in all cases (21). The 11 AD patients were either APOE4 homozygotes (n = 5), APOE3-4 heterozygotes (n = 2), or APOE3 homozygotes (n = 4). The 6 control subjects were either APOE3 homozygotes (n = 3) or APOE3-4 heterozygotes (n = 3). A single APOE2 heterozygote was available for study. Neither APOE2 homozygotes nor age-matched nondemented APOE4 homozygotes were available for study.

The lipoprotein fraction (d < 1.210 g/ml) was isolated from 8.5–10 ml of CSF by ultracentrifugation and washed once. In the two patients from 1998, CSF was directly aspirated into the thiol trapping agent 5,5′-dithiobis(2-nitrobenzoate) (DTNB) according to published methods (22). For these samples, lipoprotein fractions were prepared and dialyzed in the presence of DTNB and compared to the same patient's CSF lipoproteins collected and prepared without DTNB. After dialysis to remove salt (DTNB-containing samples were dialyzed in the presence of DTNB), protein concentration was determined by the bicinchoninic method. The lipoprotein fraction was lyophilized and delipidated using ethanol/ether. Proteins were analyzed by SDS-PAGE under reducing and nonreducing conditions, and stained with silver nitrate or immunoblotted for apoE, apoA-I, apoA-II, or apoB (20). Silver nitrate-stained gels and apoA-II immunoblots were quantified under linear conditions established by serial dilutions of CSF d < 1.210 g/ml fractions or human recombinant apoA-II using a Bio-Rad GS-700 Imaging Densitometer with Molecular Analyst software as previously described (4). Partial amino acid sequencing was performed on proteins electroblotted to PVDF membranes as described (23). Proteins were electroeluted from gels using an Amicon Centrilutor according to the manufacturer's specifications.

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections of hippocampus and superior temporal gyrus from four AD patients and three control subjects according to previously published methods (24). Immunohistochemistry also was performed on sections of human liver from two different patients with no known hepatic disease and no pathological lesions identified postmortem. Liver tissue had been formalin-fixed and embedded in paraffin the same as brain tissue. Anti-apoE antiserum was used at 1:1000 dilution and anti-apoA-II antiserum was used at 1:10,000 dilution in immunohistochemistry experiments. Competition experiments used anti-apoA-II at 1:10,000 dilution and human recombinant apoA-II at 1 mg/ml.

Values between AD patients and control subjects were compared by Student's *t*-test and values among patients with different APOE alleles were compared with Spearman ranked correlations using Prism 2.0, GraphPad Software Inc., San Diego, CA.

RESULTS

CSF apolipoprotein composition in individuals with different APOE genotypes

A lipoprotein fraction (d < 1.210 g/ml) was purified from human CSF by ultracentrifugation. No d < 1.210 g/ml fraction had detectable apoB by immunoblotting, indicating that CSF aspirates were free from contamina-

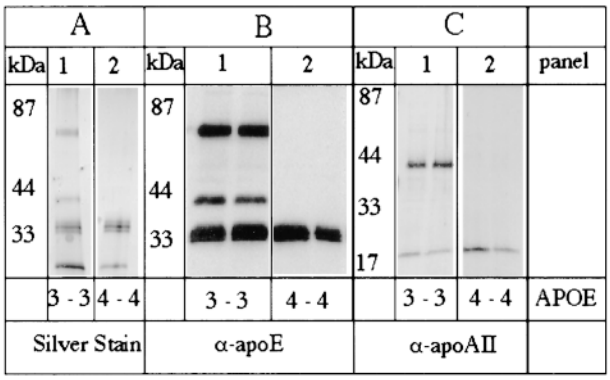


Fig. 1. CSF apolipoproteins in patients with different APOE genotypes. CSF d < 1.210 g/ml fraction from APOE3 (panel 1) and APOE4 (panel 2) homozygotes was separated by SDS-PAGE under nonreducing conditions. A: Silver nitrate staining of a 10% gel. B: Anti-apoE immunoblot of a 10% gel transferred to Immunlite membrane. Two different subjects of each APOE genotype are presented. C: Anti-apoA-II immunoblot of a 10–20% linear gradient gel transferred to Immunlite membrane. The same subjects are presented as in blot B.

tion by blood (not shown). **Figure 1** shows representative results from APOE3 and APOE4 homozygotes. Under non-reducing conditions, SDS-PAGE followed by silver nitrate staining revealed either two or four major protein bands in the d < 1.210 g/ml fraction depending on the APOE allele (Fig. 1A). In APOE3 homozygotes, four bands were present with approximate molecular masses of 69, 43, 34, and 27 kDa. In APOE4 homozygotes, only two bands were present with approximate molecular masses of 34 and 27 kDa. APOE3-4 heterozygotes had a banding pattern similar to APOE3 homozygotes (not shown). Under reducing conditions, the protein banding pattern of all d < 1.210 g/ml samples, regardless of APOE genotype, simplified to the 34 kDa and 27 kDa bands (as in Fig. 1A, lane 2), indicating that the 69 and 43 kDa species were disulfide-linked dimers. There were no differences in the amounts of these disulfide-linked dimers of apoE by silver-stained SDS-PAGE gels or anti-apoE immunoblots when samples were collected and prepared in the presence or absence of the thiol-trapping agent DTNB, indicating that the apoE-containing disulfide-linked dimers did not form ex vivo. Moreover, the d > 1.210 g/ml fraction of CSF contained less than 10% of each of these 4 proteins compared to the d < 1.210 g/ml fraction from the same patient.

Western blot analysis was then performed on nonreduced d < 1.210 g/ml fractions from CSF. Anti-apoA-I antiserum was immunoreactive with the 27 kDa band (not shown). Anti-apoE immunoreactivity was observed in the 34 kDa band in all samples (Fig. 1B). The 43 kDa and 69 kDa bands present in APOE3 homozygotes also were immunoreactive with anti-apoE antisera. ApoE3 is known to form a disulfide-linked homodimer in plasma and CSF (11). Therefore tentative assignments were made for three of the bands: the 27 kDa protein was A-I, the 34 kDa protein was apoE monomer, the 69 kDa protein was apoE dimer. These results agreed well with data from others (2–4).

As a 43 kDa apoE–apoA-II disulfide-linked heterodimer has been identified in human plasma from patients with unestablished APOE genotypes (25), we hypothesized that the 43 kDa protein complex in CSF lipoproteins from people with an APOE3 might also be apoE–apoA-II. Western blot analysis using anti-apoA-II antiserum demonstrated two apoA-II immunoreactive species in nonreduced d < 1.210 g/ml fraction (Fig. 1C); these migrated with approximate molecular masses of 18 kDa and 43 kDa. Human plasma apoA-II is known to exist as an approximately 18 kDa homodimer (26). Both bands were present in APOE3 homozygotes; however, only the 18 kDa band was present in APOE4 homozygotes. Reduction of CSF d < 1.210 g/ml fraction shifted apoA-II immunoreactivity to an 8 kDa species (as in Fig. 2C, lane 2). Therefore, tentative assignments for the 43 kDa and 18 kDa proteins were apoE3–apoA-II and apoA-II homodimer, respectively.

In order to identify definitively the 43 kDa protein, CSF lipoproteins of two APOE3 homozygotes were separated by electrophoresis and the 43 kDa band was excised, electroeluted, concentrated, and subjected to further electrophoresis. **Figure 2** shows the results for one of these individuals. Silver staining under nonreducing conditions showed one protein band that migrated at 43 kDa (Fig. 2A, lane 2). Immunoblotting established that this protein band was immunoreactive with both anti-apoE and anti-apoA-II antisera (Fig. 2B, lane 2, and Fig. 2C, lane 1, respectively). Reducing conditions yielded a 34 kDa protein immunoreactive with anti-apoE antiserum (Fig. 2B, lane 3) and an 8 kDa protein immunoreactive with anti-apoA-II antiserum (Fig. 2C, lane 2). Identical results were obtained from the other APOE3 homozygote (not shown).

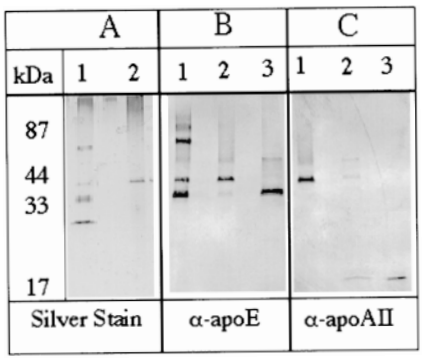


Fig. 2. Identification of the 43 kDa protein complex in CSF of APOE3 homozygotes. CSF d < 1.210 g/ml fraction was separated by SDS-PAGE and the 43 kDa species was purified by excision, electroelution, and concentration. All gels were 10–20% linear gradients. A: Silver nitrate stain of original nonreduced d < 1.210 g/ml fraction (lane 1) and nonreduced purified 43 kDa band (lane 2). B: Anti-apoE immunoblot of original nonreduced d < 1.210 g/ml fraction (lane 1), nonreduced purified 43 kDa protein (lane 2), and reduced purified 43 kDa protein (lane 3). C: Anti-apoA-II immunoblot of nonreduced purified 43 kDa protein (lane 1), reduced purified 43 kDa protein (lane 2), and reduced human recombinant apoA-II (lane 3).

Partial amino acid sequencing of the four major proteins in the d < 1.210 g/ml fraction also was performed. Nonreduced CSF d < 1.210 g/ml fractions were separated by electrophoresis, transferred to PVDF membrane, and visualized by Coomassie stain. The four major proteins from two APOE3 homozygotes were excised and subjected to N-terminal amino acid sequencing. The six N-terminal amino acids were KVEQAV for the 69 kDa and 34 kDa proteins, and DEPPQS for the 27 kDa protein, matching the known sequences for human apoE and apoA-I, respectively (26). The 43 kDa reducible complex yielded two amino acids of equal concentration per cycle: one sequence corresponded to human apoE, KVEQAV, and the remainder matched the sequence of human apoA-II, QAKEP (26). Only one amino acid, V, was detected in the sixth cycle. This was expected because the sixth amino acid in human apoA-II is C (27).

Molecular distribution of CSF apolipoproteins in AD patients and control subjects

CSF d < 1.210 g/ml fractions were prepared from 17 different individuals (**Table 1**) and assayed for the distribution of apoE- and apoA-II-containing proteins. CSF apolipoproteins were separated by SDS-PAGE, stained with silver nitrate, and the bands were quantified by densitometry (see Fig. 1A for example). Previously, we have shown that total protein concentration in the d < 1.210 g/ml fraction is significantly diminished in AD patients relative to control subjects, likely secondary to brain atrophy with increased ventricular volume and dilutional effects in AD patients (20). Moreover, we showed that despite this difference in total protein in the d < 1.210 g/ml fraction, the ratio of total apoE to apoA-I is not changed between AD and control subjects (20). Further analysis of these data revealed no significant difference in the ratio of total apoE to apoA-I as a function of APOE genotype: total apoE to apoA-I was 3.2 ± 0.5 in APOE3 homozygotes and 3.5 ± 1.1 in APOE4 homozygotes ($P > 0.05$). Thus, the ratio of total apoE to apoAI in CSF d < 1.210 g/ml fraction is constant with respect to the presence or absence of AD and inheritance of APOE3 or APOE4. Therefore, the integrated peak area of each apoE-containing apolipoprotein was expressed relative to that of apoA-I to control for dilutional effects of brain atrophy and variation in sample preparation among patients.

TABLE 1. Clinical and pathological information on the 17 individuals included in this study

Subjects	n	Age	Female to Male	Postmortem Interval	Brain Weight
		yr		*h*	*g*
AD Patients	9	77.4 ± 2.2	5:4	3.0 ± 0.4	1049 ± 54
Control	6	81.0 ± 2.6	3:3	2.8 ± 0.3	1255 ± 48[a]

Values are given as mean ± SEM. Age at death, gender ratio, postmortem interval, and unfixed brain weight are presented. The only variable significantly different between AD and control subjects was brain weight ([a]$P < 0.05$).

In a subset of subjects with the same APOE genotypes (APOE3 homozygotes or APOE3-4 heterozygotes), the presence or absence of AD did not appear to alter significantly the relative amounts of the three apoE-containing proteins ($P > 0.05$). In contrast, there was a significant gene dosage effect of APOE on the relative levels of all three apoE-containing proteins (**Fig. 3**). Changes in the relative level of each apoE-containing protein were significantly correlated with the number of APOE3 alleles; apoE homodimer and apoE3–apoA-II increased with increasing copy number of APOE3 ($P < 0.001$) while apoE monomer decreased with increasing copy number of APOE3 ($P = 0.01$).

The low levels of apoA-II as compared to the other CSF apolipoproteins precluded quantification of different molecular forms by silver nitrate staining (see Fig.

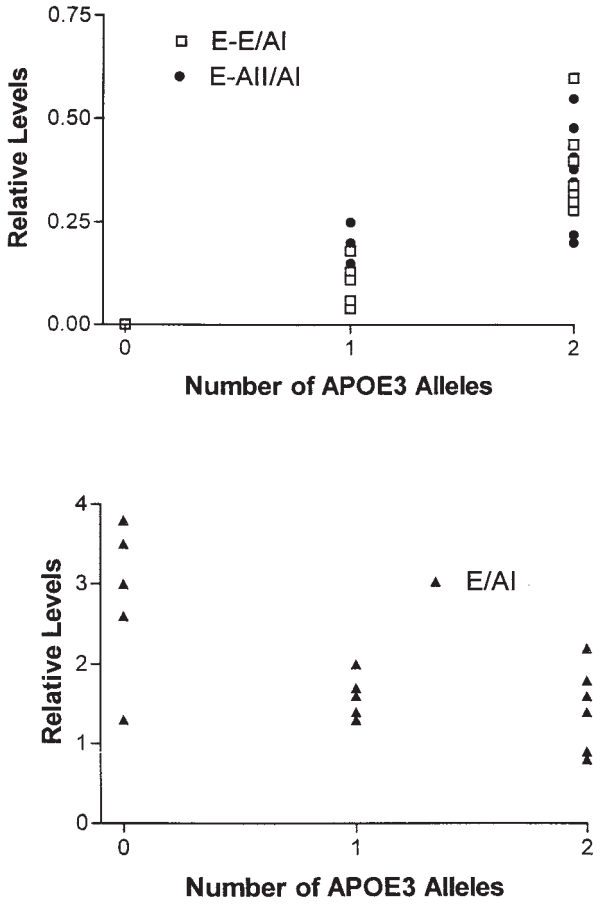


Fig. 3. Relative levels of CSF apoE-containing apolipoproteins in patients with different APOE genotypes. CSF d < 1.210 g/ml fractions from 11 AD patients and 6 control subjects. Samples were separated by 10% SDS-PAGE, stained with silver nitrate, and their band densities quantified. The integrated band density for each subject's apoE homodimer (E-E), apoE–apoA-II heterodimer (E-A-II), and apoE monomer (E) was divided by the integrated band density for apoA-I. A scatter plot was made of the resulting relative levels of each apoE-containing apolipoprotein versus the number APOE3 alleles. Note scale differences in ordinates for the two graphs. Spearman ranked correlations for relative levels versus number of APOE3 alleles were statistically significant for E-E ($r = 0.93$, $P < 0.001$), E-A-II ($r = 0.88$, $P < 0.001$), and E ($r = -0.51$, $P < 0.05$).

2A, lane 1). For this reason, we assessed the distribution of apoA-II using immunoblots probed with anti-apoA-II antiserum. Figure 1C illustrates typical results from four different subjects. The relative distribution of apoA-II immunoreactivity in apoE3–apoA-II heterodimer and apoA-II homodimer was computed for each subject (**Fig. 4**). Again, there was a significant APOE3 gene dosage effect; the percentage of A-II as homodimer or apoE3–apoA-II heterodimer significantly changed with increasing copy number of APOE3 ($P < 0.001$).

Our only patient with an APOE2 was a neurologically normal woman (87 years), not included in the above dataset. She was APOE heterozygous with APOE2 and APOE3 alleles. This subject displayed yet another protein band in her CSF d < 1.210 g/ml fraction that was not observed in APOE3 homozygotes (not shown). This additional protein was approximately 50 kDa and was immunoreactive with both anti-apoE and anti-apoA-II antisera. Others have identified apoE2-$(apoA\text{-}II)_2$ in human plasma (28), and, therefore, we also provisionally classify this CSF apolipoprotein as apoE2-$(apoA\text{-}II)_2$.

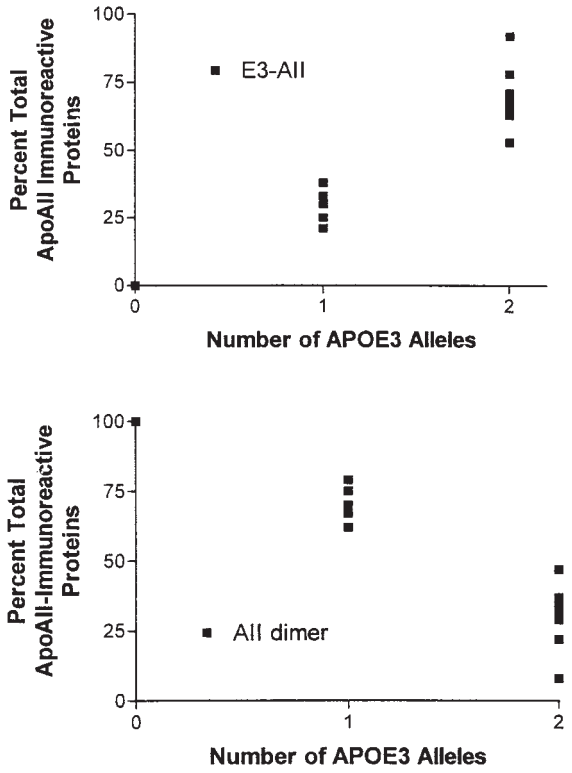


Fig. 4. Molecular distribution of apoA-II in CSF from patients with different APOE genotypes. CSF d < 1.210 g/ml fractions from 11 AD patients and 6 control subjects. Samples were separated by 10–20% linear gradient SDS-PAGE, transferred to membrane, immunoblotted with anti-apoA-II antiserum, and the band density quantified. The percentage of integrated band density present as apoA-II homodimer or apoE–apoA-II was graphed as a scatter plot versus the number of APOE3 alleles. Spearman ranked correlations for percent total immunoreactive protein versus number of APOE3 alleles were statistically significant for E-A-II ($r = 0.95$, $P < 0.001$) and A-II-A-II ($r = -0.95$, $P < 0.001$).

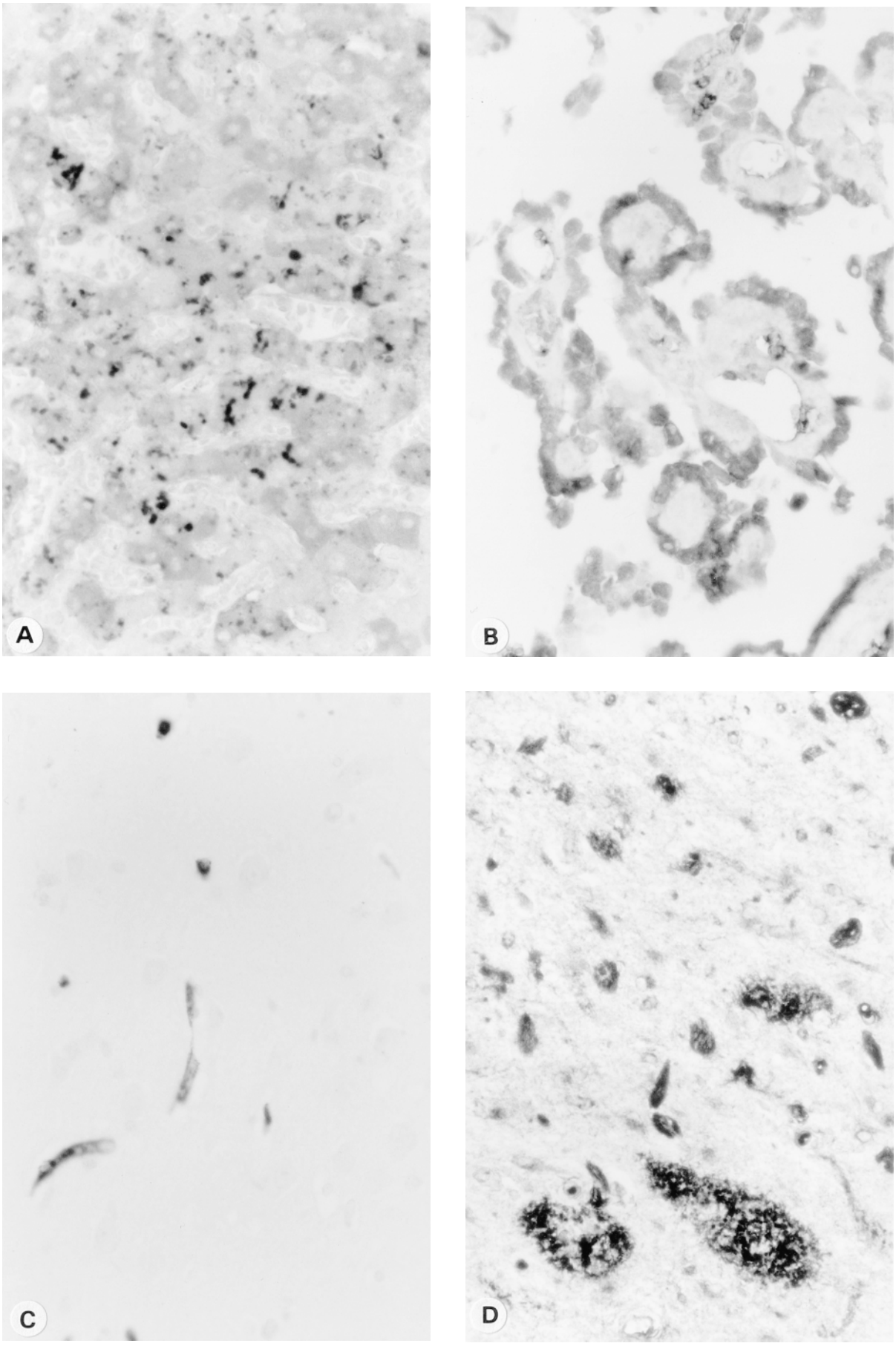
ApoA-II immunohistochemistry of human brain tissue from AD patients and control subjects

ApoA-II immunohistochemical analysis was performed on tissue sections of human liver, cerebral cortex, and hippocampus. Tissue sections of liver from three control subjects (aged 73 to 86 years) were examined. Vesicular apoA-II immunoreactivity was present in hepatocyte cytoplasm (**Fig. 5**), a pattern consistent with synthesis and secretion of apoA-II by hepatocytes. ApoA-II immunoreactivity was completely blocked in hepatocytes by preincubation of antiserum with human recombinant apoA-II. Tissue sections of brain from three control subjects as well as four patients with AD (aged 68 to 85 years, two APOE3 homozygotes and two APOE4 homozygotes) also were examined. In all seven individuals, apoA-II immunoreactivity was present only in the cytoplasm of choroid plexus epithelium as well as intravascular immunoreactivity, a pattern consistent with transport into CSF but not with synthesis in brain tissue. ApoA-II immunoreactivity in brain sections also was ablated by preincubation of antiserum with human recombinant apoA-II. Importantly, no apoA-II immunoreactivity was observed with neuritic plaques or neurofibrillary tangles even in the AD APOE3 homozygotes. In contrast, consecutive tissue sections from the same AD patients demonstrated apoE immunoreactivity with numerous neuritic plaques and neurofibrillary tangles in both APOE3 and APOE4 homozygotes, as has been observed by others (29). Thus, despite a significant fraction of apoE being present as apoE–apoA-II in APOE3 homozygotes and extensive apoE immunoreactivity with plaques and neurofibrillary tangles, no apoA-II immunoreactivity was identified on these structures.

DISCUSSION

Using the largest series of human subjects to date, we corroborated that apoE and apoA-I are the major apolipoproteins in human CSF. In addition, despite previously conflicting reports, we have conclusively demonstrated the presence of apoA-II in human CSF. Moreover, we definitively identified a CSF apolipoprotein complex formed in vivo, apoE3–apoA-II. We showed that the relative levels of all apoE-containing CSF apolipoproteins as well as the molecular distribution of CSF apoA-II are significantly related to APOE genotype but not to AD. Finally, immunohistochemical data indicated that apoA-II is not synthesized at detectable levels by human brain, and that, unlike apoE, pathological structures in AD brain are not apoA-II immunoreactive.

Although it may seem trivial that apoE–apoA-II found in plasma also exists in CSF lipoproteins, there are valid reasons to expect this might not happen in CSF. Human CNS apoE is derived from a different source than is plasma apoE and the two pools do not mix (2, 3, 6, 7, 10). Also, Northern blot data of others and our immunohistochemical data indicate that apoA-II is not synthesized in brain (30). Thus, our demonstration of apoE–apoA-II het-



erodimer in CSF lipoproteins suggests that astrocyte-derived apoE and plasma-derived apoA-II interact in the CNS. A possible explanation for why apoE3–apoA-II has not been identified previously in CNS is that of the species studied only humans, rabbits, and cows have cysteinyl-containing apoE, and only humans and chimpanzees have cysteinyl-containing apoA-II (10). Therefore, a disulfide-linked heterodimer of apoE and apoA-II would be present only in humans.

Several previous studies have measured total apoE levels immunochemically in human lumbar CSF aspirates from control subjects and patients with AD (17–19). Some have observed reduced CSF apoE concentration in AD patients as compared with controls, while others have not observed this difference between groups. Those studies that did detect lower total CSF apoE levels have shown that it was not related to APOE genotype. We have demonstrated that the overall apolipoprotein concentration in CSF is reduced in AD patients relative to age-matched controls, while the ratio of total apoE to apoA-I remains unchanged between AD and control subjects as well as among patients with different APOE genotypes (20). Given that the average decrease in overall CSF apolipoprotein concentration in AD patients is equivalent to the average increase in ventricular volume secondary to brain atrophy in AD, we interpret this general reduction in apolipoprotein concentration to be due largely to dilutional effects from brain atrophy in AD patients.

In order to control for dilutional effects and any differences in sample preparation, we directly quantified individual apolipoproteins with silver nitrate staining and then expressed levels of apoE-containing apolipoproteins relative to the level of apoA-I. It is important to note that as different proteins bind different amounts of silver stain, these ratios are indices of relative levels of apoE-containing apolipoproteins and cannot be interpreted as mass ratios of apolipoproteins. Using this approach, we found that the relative levels of each individual apoE-containing protein displayed significant APOE allele dosage effects. Recently, another group has identified apoA-II immunochemically in human CSF and noted the co-localization of apoE and apoA-II immunoreactivity in Western blots of human CSF $d < 1.210$ g/ml fraction. This group quantified the percentage of apoE immunoreactivity as dimers in AD patients' CSF, but not control subjects', and obtained results very similar to ours (9).

As an initial assessment of the neurobiological significance of apoA-II-containing proteins, we performed anti-apoA-II immunohistochemistry on human cerebral cortical and hippocampal tissue sections from AD patients homozygous for APOE3 or APOE4 and age-matched control subjects. ApoA-II immunoreactivity was detectable only in the cytoplasm of choroid plexus epithelium and within blood vessels but not in any other normal or pathological structure. Thus, apoA-II appears similar to apoA-I in that it is synthesized and secreted primarily by hepatocytes and enterocytes, but not in brain parenchyma, and then transported into CSF perhaps in part associated with HDL. It is noteworthy that apoAII immunoreactivity was not observed with senile plaques or neurofibrillary tangles in AD patients; both of these structures were strongly immunoreactive for apoE. ApoE binding to the extracellular senile plaques is thought to occur via the low density lipoprotein receptor-related protein or LRP (31). Lack of apoA-II immunoreactivity in APOE4 homozygotes suggests that apoA-II homodimer lacks affinity for these pathological structures. In APOE3 homozygotes, a substantial fraction of apoE exists as an apoE3–apoA-II heterodimer, yet there still was no apoA-II immunoreactivity. As others have reported that apoE–apoA-II has greatly diminished LDL receptor binding compared to apoE (32), one interpretation of these data is that formation of the apoE–apoA-II complex alters the binding characteristics of apoE for these pathological structures in AD brain.

The risk for AD has been statistically associated with inheritance of APOE4, yet the mechanisms underlying this genetic association remain unknown. Considerable effort has been devoted to understanding the neurobiological effects of apoE isoforms in neuronal culture and alteration of βA metabolism by apoE (33–41). Alternatively, we have shown that the tissue distribution of neurotoxic lipid peroxidation products in diseased regions of AD brain is significantly associated with APOE genotype (24, 42, 43). Specifically, we have shown that protein adducts of 4-hydroxynonenal accumulate exclusively in pyramidal neurons of AD APOE4 homozygotes but are dispersed among neurons and glia in AD APOE3 homozygotes. Recently our laboratory has shown that the lipid constituents of CSF lipoproteins are significantly modified in AD patients relative to age-matched controls and that these changes are indicative of extensive lipid peroxidation; however, these modifications are independent of APOE genotype (20).

Others have shown that apoE isoforms differ significantly in their receptor binding activity; apoE2 has two orders of magnitude less LDL receptor binding affinity than apoE3 or apoE4, and apoE3 dimers have greatly diminished LDL receptor binding activity compared to apoE monomer (10, 22, 32). Coupled with our results showing that a significant fraction of CNS apoE3 exists as dimers, one may speculate that the apoE receptor binding activity in human CNS is inversely related to the APOE genetic susceptibility for AD. Given that in vitro studies have shown that CNS lipoproteins are delivered to neurons via apoE-dependent mechanisms, that oxidized lipoproteins may be neurotoxic, and that CNS lipoproteins are oxi-

Fig. 5. ApoA-II and apoE immunohistochemistry. Photomicrographs of tissue sections analyzed by anti-apoA-II or anti-apoE immunohistochemistry and then stained with hematoxylin (all ×400). Anti-apoA-II immunohistochemistry of tissue sections of human liver (A), choroid plexus (B), and CA1 of hippocampus from an AD patient (C). Anti-apoE immunohistochemistry of CA1 of hippocampus from the same AD patient as in C that shows extensive immunoreactivity with plaques and neurofibrillary tangles (D).

dized in AD patients (20, 44), we propose that mechanisms which diminish apoE-mediated delivery of neurotoxic oxidized lipoproteins, e.g., inheritance of APOE2 or formation of apoE dimers, may be neuroprotective and contribute to the stratification of risk for AD with APOE genotype.

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REFERENCES

1. Swahn, B., R. Bronnestam, and S. J. Dencker. 1961. On the origin of the lipoproteins in the cerebrospinal fluid. *Neurology.* **11:** 437–440.
2. Roheim, P. S., M. Carey, T. Forte, and G. L. Vega. 1979. Apolipoproteins in human cerebrospinal fluid. *Proc. Natl. Acad. Sci. USA.* **76:** 4646–4649.
3. Pitas, R. E., J. K. Boyles, S. H. Lee, D. Hui, and K. H. Weisgraber. 1987. Lipoproteins and their receptors in the central nervous system. *J. Biol. Chem.* **262:** 14352–14360.
4. Borghini, I., F. Barja, D. Pometta, and R. James. 1995. Charaterization of subpopulations of lipoprotein particles isolated from human cerebrospinal fluid. *Biochim. Biophys. Acta.* **1225:** 192–200.
5. Elshourbagy, N. A., W. S. Liao, R. W. Mahley, and J. M. Taylor. 1985. Apolipoprotein E mRNA is abundant in the brain and adrenals, as well as in the liver, and is present in other peripheral tissues of rats and marmosets. *Proc. Natl. Acad. Sci. USA.* **82:** 203–207.
6. Linton, M., R. Gish, S. T. Hubl, E. Butler, C. Esquivel, W. I. Bry, J. K. Boyles, M. R. Wardell, and S. G. Young. 1991. Phenotypes of apolipoprotein B and apolipoprotein E after liver transplantation. *J. Clin. Invest.* **88:** 270–281.
7. Pitas, R., J. Boyles, S. Lee, D. Foss, and R. Mahley. 1987. Astrocytes synthesize apolipoprotein E and metabolize apolipoprotein E-containing lipoproteins. *Biochim. Biophys. Acta.* **917:** 148–161.
8. DeVries, H., B. Breedveld, J. Kuiper, A. DeBoer, T. VanBerkel, and D. Breimer. 1995. High-density lipoprotein and cerebral endothelial cells in vitro: interactions and transport. *Biochem. Pharmacol.* **50:** 271–273.
9. Rebeck, G. W., N. C. Alonzo, O. Berezovska, S. D. Harr, R. B. Knowles, J. H. Growdon, B. T. Hyman, and A. J. Mendez. 1998. Structure and function of human cerebrospinal fluid lipoproteins from individuals of different APOE genotypes. *Exp. Neurol.* **149:** 175–182.
10. Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science.* **240:** 622–630.
11. Weisgraber, K. H. 1994. Apolipoprotein E: structure–function relationships. *Adv. Protein Chem.* **45:** 249–302.
12. Roses, A. 1995. On the metabolism of apolipoprotein E and the Alzheimer diseases. *Exp. Neurol.* **132:** 149–156.
13. Strittmatter, W. J., and A. D. Roses. 1995. Apolipoprotein E and Alzheimer's disease. *Proc. Natl. Acad. Sci. USA.* **92:** 4725–4727.
14. Alberts, M., C. Graffagnino, C. McClenny, D. DeLong, W. Strittmatter, A. Saunders, and A. Roses. 1995. APOE genotype and survival from intracerebral hemorrhage. *Lancet.* **346:** 575.
15. Newman, M., N. Croughwell, J. Blumenthal, E. Lowry, W. White, W. Spillane, R. Davis, D. Glower, L. Smith, and E. Mahanna. 1995. Predictors of cognitive decline after cardiac operation. *Ann. Thorac. Surg.* **59:** 1326–1330.
16. Nicoll, J., G. Roberts, and D. Graham. 1995. Apolipoprotein E epsilon 4 allele is associated with deposition of amyloid beta-protein following head injury. *Nature Med.* **1:** 135–137.
17. Blennow, K., C. Hesse, and P. Fredman. 1994. Cerebrospinal fluid apolipoprotein E is reduced in Alzheimer's disease. *Neuroreport.* **5:** 2534–2536.
18. Landen, M., C. Hesse, P. Fredman, B. Regland, A. Wallin, and K. Blennow. 1996. Apolipoprotein E in cerebrospinal fluid from patients with Alzheimer's disease and other forms of dementia is reduced but without any correlation to the apoE4 isoform. *Dementia.* **7:** 273–278.
19. Rosler, N., I. Wichart, and K. Jellinger. 1996. Intra vitam lumbar cerebrospinal fluid and serum and postmortem ventricular immunoreactive apolipoprotein E in patients with Alzheimer's disease. *J. Neurol. Neurosurg. Psych.* **60:** 452–454.
20. Montine, T. J., K. S. Montine, and L. L. Swift. 1997. Central nervous system lipoproteins in Alzheimer's disease. *Am. J. Pathol.* **151:** 1571–1575.
21. Saunders, A. M., W. J. Strittmatter, D. Schmechel, P. H. St. George-Hyslop, S. H. Joo, B. L. Rosi, J. F. Gusella, M. D. Crapper, M. J. Alberts, C. Hulette, B. Crain, D. Goldgaber, and A. D. Roses. 1993. Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease. *Neurology.* **43:** 1467–1472.
22. Weisgraber, K. H., and L. H. Shinto. 1991. Identification of the disulfide-linked homodimer of apolipoprotein E3 in plasma. Impact on receptor binding activity. *J. Biol. Chem.* **266:** 12029–12034.
23. Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262:** 10035–10038.
24. Montine, K., S. Olson, V. Amarnath, W. Whetsell, D. Graham, and T. Montine. 1997. Immunochemical detection of 4-hydroxynonenal adducts in Alzheimer's disease is associated with APOE4. *Am. J. Pathol.* **150:** 437–443.
25. Weisgraber, K. H., and R. W. Mahley. 1978. Apoprotein (E-AII) complex of human plasma lipoproteins. I. Characterization of this mixed disulfide and its identification in a high density lipoprotein subfraction. *J. Biol. Chem.* **253:** 6281–6288.
26. Mahley, R. W., T. L. Innerarity, S. C. Rall, and K. H. Weisgraber. 1984. Plasma lipoproteins: apolipoprotein structure and function. *J. Lipid Res.* **25:** 1277–1294.
27. Findlay, J. B., and M. J. Geisow. 1989. Protein Sequencing. A Practical Approach. IRL Press, New York. 199.
28. Tozuka, M., H. Hidaka, M. Miyachi, K. Furihata, T. Katsuyama, and M. Kanai. 1992. Identification and characterization of apolipoprotein(AII-E2-AII) complex in human plasma lipoprotein. *Biochim. Biophys. Acta.* **1165:** 61–67.
29. Namba, Y., M. Tomonaga, H. Kawasaki, E. Otomo, and K. Ikeda. 1991. Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and kuru plaque amyloid in Creutzfeldt-Jakob disease. *Brain Res.* **541:** 163–166.
30. Srivastava, R. A., N. Srivastava, and G. Schonfeld. 1992. Expression of low density lipoprotein receptor, apolipoprotein A-I, A-II, and A-IV in various rat organs utilizing an efficient and rapid method for RNA isolation. *Biochem. Int.* **27:** 85–95.
31. Rebeck, G. W., S. D. Harr, D. K. Strickland, and B. T. Hyman. 1995. Multiple, diverse senile plaque-associated proteins are ligands of an apolipoprotein E receptor, the alpha 2-macroglobulin receptor/low-density-lipoprotein receptor-related protein, *Annal. Neurol.* **37:** 211–217.
32. Innerarity, T. L., R. W. Mahley, K. H. Weisgraber, and T. P. Bersot. 1978. Apoprotein (E-AII) complex of human plasma lipoproteins II. Receptor binding activity of a high density lipoprotein subfraction modulated by the apo(E-AII) complex. *J. Biol. Chem.* **253:** 6289–6295.
33. Bellosta, S., B. Nathan, M. Orth, L. Dong, R. Mahley, and R. Pitas. 1995. Stable expression and secretion of apolipoproteins E3 and E4 in mouse neuroblastoma cells produces differential effects on neurite outgrowth. *J. Biol. Chem.* **270:** 27063–27071.
34. Fagan, A., G. Bu, Y. Sun, A. Daugherty, and D. Holtzman. 1996. Apolipoprotein E-containing high density lipoprotein promotes neurite outgrowth and is a ligand for the low density lipoprotein receptor-related protein. *J. Biol. Chem.* **271:** 30121–30125.
35. Gutman, C. R., W. J. Strittmatter, K. H. Weisgraber, and W. D. Matthew. 1997. Apolipoprotein E binds and potentiates the biological activity of ciliary neurotrophic factor. *J. Neurosci.* **17:** 6114–6121.
36. Holtzman, D., R. Pitas, J. Kilbridge, B. Nathan, R. Mahley, G. Bu, and A. Schwartz. 1995. Low density lipoprotein receptor-related protein mediates apolipoprotein E-dependent neurite outgrowth in central nervous system-derived neuronal cell line. *Proc. Natl. Acad. Sci. USA.* **92:** 9480–9484.
37. Huang, D., K. Weisgraber, W. Strittmatter, and W. Matthew. 1995. Interaction of apolipoprotein E with laminin increases neuronal

adhesion and alters neurite morphology. *Exp. Neurol.* **136:** 251–257.
38. Nathan, B., K. Chang, S. Bellosta, E. Brisch, G. Nianfeng, R. Mahley, and R. Pitas. 1995. The inhibitory effect of apolipoprotein E4 on neurite outgrowth is associated with microtubule depolymerization. *J. Biol. Chem.* **270:** 19791–19799.
39. Nathan, B., S. Bellosta, D. Sanan, K. Weisgraber, R. Mahley, and R. Pitas. 1994. Differential effects of apolipoproteins E3 and E4 on neuronal growth in vitro. *Science.* **264:** 850–852.
40. Miyata, M., and J. D. Smith. 1996. Apolipoprotein E allele-specific antioxidant activity and effects on cytotoxicity by oxidative insults and beta-amyloid peptides. *Nature Genet.* **14:** 55–61.
41. Montine, T. J., D. Y. Huang, W. M. Valentine, V. Amarnath, A. Saunders, K. H. Weisgraber, D. G. Graham, and W. J. Strittmatter. 1996. Crosslinking of apolipoprotein E by products of lipid peroxidation. *J. Neuropathol. Exp. Neurol.* **55:** 202–210.
42. Montine, K. S., P. J. Kim, S. J. Olson, W. R. Markesbery, and T. J. Montine. 1997. 4-Hydroxy-2-nonenal pyrrole adducts in human neurodegenerative disease. *J. Neuropathol. Exp. Neurol.* **56:** 866–871.
43. Montine, K., E. Reich, S. J. Olson, W. R. Markesbery, and T. Montine. 1998. Distribution of reducible 4-hydroxynonenal adduct immunoreactivity in Alzheimer's disease is associated with APOE genotype. *J. Neuropathol. Exp. Neurol.* **57:** 415–425.
44. Kivatinitz, S. C., M. A. Pelsman, A. C. Alonso, L. Bagatolli, and S. Quiroga. 1997. High density lipoprotein aggregated by oxidation induces degeneration of neuronal cells. *J. Neurochem.* **69:** 2102–2114.