Neurons of the Human Frontal Cortex Display Apolipoprotein E Immunoreactivity: Implications for Alzheimer’s Disease

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Abstract. Apolipoprotein E (apoE) is a plasma protein that regulates lipid transport and cholesterol homeostasis. In humans, apoE occurs as 3 major isoforms (apoE2, E3, and E4). Genetic evidence demonstrates an overrepresentation of the apoE ε4 allele in Alzheimer’s disease (AD). While apoE immunoreactivity (IR) is associated with the amyloid plaques and neurofibrillary tangles of AD, few studies have characterized the localization of apoE in normal human brains. We examined the distribution of apoE in the cerebral cortex of normal aged individuals and compared the results to clinically diagnosed and pathologically confirmed AD cases. In addition, we characterized the apoE IR in brains from high plaque non-demented (HPND) cases. We observed consistent and widespread apoE staining in cortical neurons from normal and HPND individuals. This finding was confirmed by double immunostaining which colocalized apoE with microtubule-associated protein-2, as well as low density lipoprotein receptor-related protein, an apoE receptor found on neurons. In contrast, AD brains displayed apoE IR in plaques and neurofibrillary tangles with little neuronal staining. This data clearly establishes the presence of apoE in normal neurons, supporting an intracellular role for apoE. Moreover, the results suggest that this function of apoE is disrupted in AD, where apoE staining of neurons was drastically reduced.

Key Words: Alzheimer’s disease; Apolipoprotein E; β-amyloid; Human cerebral cortex; Immunohistochemistry; Low density lipoprotein receptor-related protein; Microtubule-associated proteins.

INTRODUCTION

Apolipoprotein E (apoE) is a 35-kDa lipophilic protein that circulates in the plasma associated with several classes of lipoproteins. In humans, apoE occurs as 3 major isoforms, E2 (Cys112, Cys158), E3 (Cys112, Arg118), and E4 (Arg112, Arg122), which are products of allelic variations at a single genetic locus. As a ligand for lipoprotein receptors, including low density lipoprotein (LDL) receptor and LDL receptor-related protein (LRP), apoE regulates lipid transport and cholesterol homeostasis.

In addition to its role in dietary lipid transport and metabolism, apoE also has functions in the nervous system. The major site of apoE synthesis is the liver; however, the brain contains the second highest abundance of apoE mRNA (1). By immunohistochemistry, the cellular distribution of apoE in the brain was found primarily in astroglia (2–5), though apoE staining of neurons was also reported in several cases (6, 7). Immunostaining has also identified apoE receptors in the brain. LDL receptor immunostaining is localized to astrocytes (4, 8) and LRP is localized to neurons (4, 9) and activated astrocytes (10). In cultured neurons, the addition of apoE promotes neurite outgrowth, a process mediated by interactions with apoE receptors (11, 12). Thus, the capacity of brain tissue for apoE synthesis, uptake, or both is important to normal neuronal function.

ApoE also has a role in neural pathology. ApoE mRNA levels increase in response to injury in both the peripheral (13) and central (14) nervous systems, as well as in the brains of Alzheimer’s disease (AD) patients (6). There is compelling genetic evidence demonstrating an overrepresentation of the apoE ε4 allele in subjects with sporadic (15) and late-onset familial AD (16). ApoE immunostains the classic hallmarks of AD brains: extracellular senile plaques (SPs) containing β-amyloid (Aβ), and intracellular neurofibrillary tangles (NFTs) containing phosphorylated tau protein assembled as paired helical filaments (PHFs) (17). In addition, the density of Aβ deposition in cerebral plaques of patients with late-onset AD positively correlates with the ε4 allele (4, 18). While it is now clear that ε4 is a risk factor for AD, the exact mechanism by which apoE exerts its isoform-specific effects on the etiology of this disease remains unknown.

In spite of genetic, histochemical, and biochemical evidence demonstrating a role for apoE in both normal and pathological neuronal function, to date few studies have characterized the cellular localization of apoE in normal human brains. Therefore the aim of this study is to localize apoE in non-diseased as well as in AD brains.

MATERIALS AND METHODS

Case Material

Brain tissues were obtained from the Rush Alzheimer’s Center at Rush Presbyterian St. Luke’s Medical Center and were processed as previously described (19). Briefly, the diagnosis of AD was based on clinical history of dementia and neuropathological evaluations using the age adjusted NIA/ADRDA
FRONTAL CORTICAL NEURONS ARE APOE IMMUNOPositIVE

(20) and the CERAD (21) criteria. The absence of dementia in control cases was determined by retrospective examination of medical records, as well as through interviews with physicians and family of subjects. The superior frontal cortex was collected from 10 AD patients (mean age ± SD: 77 ± 7) and 10 elderly individuals that served as normal controls (NC) (mean age ± SD: 72 ± 13). In addition, brains were collected from 3 individuals (mean age ± SD: 79 ± 2) who showed no clinical evidence of dementia but had high numbers of senile plaques (HPND). NC, HPND, and AD brain weights (grams ± SD: 1,250 ± 199, 1,183 ± 72, and 1,162 ± 116, respectively) and postmortem intervals (hours ± SD: 13 ± 7, 13 ± 2, and 9 ± 6, respectively) were similar. Cortical samples were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.4 (PB) for 24 to 48 hours (h) at 4°C, cryoprotected in graded concentrations of sucrose solutions (10 to 30%) in PB, and sectioned frozen at 40 μm on a sliding microtome. Sections were collected serially and stored at -20°C in a cryoprotectant solution containing ethylene glycol-glycerol-phosphate buffer (22).

ApoE Genotyping

Genomic DNA was isolated from brain tissues by Proteinase-K digestion of the tissue and isopropanol precipitation of the DNA (23). ApoE genotypes (ε2, ε3, ε4) of DNA samples were determined by the polymerase chain reaction (PCR) procedure previously described (24) using 2.5 U of AmpliTaq® polymerase (Perkin-Elmer Cetus) per reaction. The resulting 244 bp product was digested at 37°C for 3 h with HhaI (BRL) by adding 3 U of enzyme directly to the PCR reaction upon completion of the temperature cycling. The digested products were electrophoresed on a 4.5% MetaPhor® (FMC BioProducts) agarose gel and visualized by staining with ethidium bromide (0.5 μg/ml).

Immunochemistry

Rabbit polyclonal antisera against human apoE was prepared as previously described (25) and used at 1:800 dilution for immunohistochemical staining. Other antibodies were mouse monoclonal antibodies against GFAP (GA5 [Boehringer Mannheim], 1:1,600) LRP (8G1 [26], 1:500), and MAP-2 (AP20 [Boehringer Mannheim], 1:800). Optimal concentrations were determined empirically by staining sections with serial dilutions of the antibodies. The concentration of each antibody was chosen to provide the greatest staining intensity while maintaining low background levels. Free-floating sections were processed for single or double immunolabeling with the avidin-biotin method (27–29) with minor modifications. After washing in phosphate-buffered saline, sections were incubated for 45 minutes (min) in 0.3% H2O2 in 0.1 M Tris-buffered saline (TBS, pH 7.4), then incubated overnight in primary antibody diluted in TBS with 1% bovine serum albumin (BSA), 1% heat-inactivated normal horse or goat serum (control for secondary antibody) and 0.25% Triton X-100. Sections were subsequently incubated for 1 h in biotinylated secondary antibody (1:200) in TBS with 1% BSA. After incubation for 1 h in avidin–biotin–peroxidase complex (1:100, Vector Laboratories) in TBS, antigens were visualized by reacting with Vector SG (gray-blue color, Vector Laboratories). For double immunostaining, the first antigen was visualized with dianinozenenine (brown color, Polysciences Inc.) and the second antigen was visualized with Vector SG.

Specificity of the apoE antisera was determined by staining sections with preimmune rabbit sera, secondary antibody alone, or with apoE antibody preincubated at room temperature for 1 h with apoE (10 μg apoE protein/ml diluted antibody) purified from human serum (30).

Quantitation of ApoE Immunopositive Cells and Plaques

For maximal density determinations, apoE immunopositive neurons and plaques within a 0.25 mm2 grid were counted using a light microscope with a 16× objective. Three regions were selected on the basis of maximal neuron or plaque density. For average density determinations, a 4× objective was used to assess 8 to 30 grids (4 mm2) for the percentage of grid subdivisions (40 μm2) containing at least one immunopositive neuron or plaque. Regions were selected to span the subpial zone to cortical layer V, with at least 2 mm separating each grid. All counts were performed blind to clinical status of the cases.

Statistical Analyses

ApoE allele frequencies were analyzed using the Fisher Exact Probability test. Cell and plaque counts were analyzed using an unpaired t-test. All analyses were performed on a Macintosh® computer using the Statview 4.0 package.

RESULTS

ApoE Immunoreactivity in Normal Brain

ApoE immunohistochemistry in frontal cortex sections of normal control brains revealed intense, specific staining in neuronal cell bodies and their processes, but in few cells with astrocytic morphology. Although the size and number of the neuronal fields varied somewhat among the cases, all normal brain sections displayed considerable apoE immunoreactivity (IR) (Table 1). Typical neuronal fields were characterized by many small apoE immunopositive pyramidal cells in cortical layer III (Fig. 1A), with larger immunopositive projection neurons in layer V (Fig. 1B). The fields of apoE-stained cells in layer III were found in dense and sometimes widespread patches. ApoE staining was primarily of cell bodies with

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**TABLE 1**

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<tr>
<th>Density of ApoE Immunoreactive Neurons and Sp's in NC and AD Cerebral Cortex</th>
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<td>Neurons</td>
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<td>NC</td>
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<td>AD</td>
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* Mean (±SE) per mm2. **Percent area containing ≥1 neuron or plaque, expressed as mean ± SE. † Significantly different from NC (p < 0.05). ‡ Significantly different from NC (p < 0.01).
Fig. 1. ApoE immunostaining of NC, HPND, and AD cerebral cortex. NC sections stained with apoE antisera showing (A), fields of small pyramidal neurons in cortical layer III and (B), larger projection neurons in cortical layer V; (C) HPND section with apoE stained pyramidal neurons and SPs in cortical layer III; (D) detail of HPND section with apoE neuronal staining and immunoreactive senile plaque; (E) AD section with numerous apoE-immunoreactive SPs and no immunopositive neurons in cortical layer III. Arrows indicate apoE immunoreactive NFTs; (F) detail of AD section with an apoE-immunopositive SP; (G)
some immunopositive apical or basal dendrites. In contrast, fewer of the larger pyramidal cells seen in layer V were apoE immunoreactive. In these cells, staining intensity was comparable between the dendrites and cell bodies with faint nuclear staining. Double staining with antibodies to the neuronal marker microtubule-associated protein-2 (MAP-2) and apoE indicated that the apoE-immunopositive cells were a subset of the MAP-2 stained neurons (Fig. 2A).

Double staining of NC sections with anti-apoE and anti-glia fibrillary acidic protein (GFAP) antibodies showed no colocalization, suggesting that GFAP-positive astrocytes in the cerebral cortex do not contain abundant or detectable amounts of apoE (Fig. 2B). GFAP IR was observed primarily within astrocytes in the subpial zone of the molecular layer. Sections were also singly- and doubly-stained with antibodies to LRP and apoE/LRP. Single staining with anti-LRP antibody in frontal cortex sections showed numerous immunopositive neurons (data not shown). Similar results have been observed in human hippocampus and temporal gyrus stained for LRP (4). When NC sections were stained with anti-apoE and anti-LRP antibodies, results indicated that the apoE immunoreactive cells were a subset of the LRP stained neurons (Fig. 2C).

**ApoE Immunoreactivity in AD Brain**

In contrast to normal brain sections, there was significantly less apoE neuronal staining in AD cases (Table 1). Fewer fields of apoE immunoreactive neurons in AD sections were found, with less immunopositive cells within each field. Unlike normal controls, the apoE neuronal staining in AD brains was confined primarily to cell bodies. In addition to limited neuronal staining, AD cases also exhibited significant numbers of both diffuse and classic senile plaques with apoE IR (Table 1, Fig. 1E, F). Although we used apoE staining to quantitate the density of SPs (Table 1), values were in the same range as plaque counts based on Aβ IR (4). AD cases also had numerous NFTs with apoE IR (Fig. 1E). As with NC sections, few astrocytes were apoE immunopositive.

AD sections stained with apoE were also stained with anti-MAP-2, GFAP, or LRP antibodies. Co-staining of AD sections for MAP-2 and apoE detected MAP-2 IR in dystrophic neurites surrounding apoE immunostained plaques (Fig. 2G). In addition, although fewer neurons stained with either antibody in AD sections compared to NC, apoE stained a subset of MAP-2 positive neurons in these sections. As in NC cases, GFAP staining did not colocalize with apoE IR (Fig. 2H) but did confirm the reactive gliosis known to be associated with SPs (31). While AD sections contained few apoE or LRP immunoreactive neurons, numerous SPs contained either apoE and LRP, or LRP alone (Fig. 2I).

**ApoE Immunoreactivity in HPND Brain**

The staining characteristics of the HPND cases have similarities to both NC and AD. Although the patterns of apoE immunostained neurons in HPND were similar to NC, the plaque staining was comparable to AD (Fig. 1C, D). In HPND, the number of neurons with MAP-2 IR was less than in NC (Fig. 2D). Although MAP-2 IR was found in dystrophic neurites surrounding plaques (Fig. 2D inset), it was less prominent than in AD sections (Fig. 2G inset). As with NC and AD sections, few glial cells were apoE immunopositive. In addition, apoE and GFAP did not colocalize in HPND sections (Fig. 2E). Reactive gliosis, evidenced by GFAP staining, was less pronounced in HPND than AD cases, despite the presence of numerous SPs (Fig. 2E). A subpopulation of both LRP-stained neurons and plaques had apoE IR (Fig. 2F). These data provide evidence that HPND cases may be a preclinical state of AD, with some of the pathological markers of the disease, but no clinical symptoms. Alternatively, these individuals may be refractory to the consequences of the observed pathology.

**Specificity of ApoE Antibody**

ApoE antiserum preabsorbed with purified apoE eliminated neuronal, SP, and NFT apoE staining (Fig. 1G) and AD sections (Fig. 1H). In addition, staining with preimmune rabbit sera or secondary antibody alone produced no cellular, SP, or NFT IR (data not shown). These results indicate that staining with apoE antiserum was specific for apoE.

**ApoE Genotyping**

ApoE genotyping results from 10 NC individuals, 10 AD patients, and 3 HPND cases are summarized in Table 2. The frequency of the e4 allele in AD patients was significantly greater than in control individuals. Although derived from a relatively small sample size, the apoE allele frequencies for both control and AD groups are in agreement with earlier reports for larger populations (16, 32; Table 2).

The NC group, where only one individual was ε3/α, had the greatest apoE neuronal staining. Conversely, the AD cases had significantly less neuronal apoE and seven ε3/α genotypes. By this criteria, apoE genotype correlates with neuronal staining. However, a larger sample con-

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NC and (H) AD sections showing absence of staining in cortical layer III with apoE antiserum preabsorbed with purified apoE. Scale bar 50 μm (A–C), (E), (G), and (H). Scale bar 20 μm (D) and (F).

Fig. 2. Double immunostaining of NC, HPND, and AD cerebral cortex for apoE (blue) and MAP-2, GFAP, or LRP (brown). NC (top row), HPND (middle row), and AD (bottom row) sections are stained for apoE/MAP-2 (left column), apoE/GFAP (middle column), and apoE/LRP (right column). A. NC section showing a subset of MAP-2 stained neurons with apoE IR. Inset: Detail of apoE and MAP-2 IR colocalized to cortical neurons. D. HPND section with cortical neurons displaying apoE IR. SPs are apoE immunopositive and are associated with MAP-2 immunopositive dystrophic neurites. Inset: Detail of SP with dystrophic
TABLE 2
ApoE Allele Frequencies in NC, HPND, and AD Individuals

<table>
<thead>
<tr>
<th>ApoE Allele</th>
<th>Current study</th>
<th>Naibantoglu et al (1994)</th>
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<tr>
<td></td>
<td>NC (n = 10)</td>
<td>HPND (n = 3)</td>
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<tr>
<td>ε2</td>
<td>0.05</td>
<td>0.00</td>
</tr>
<tr>
<td>ε3</td>
<td>0.90</td>
<td>1.00</td>
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<tr>
<td>ε4</td>
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* Significantly different from NC ε4 (p < 0.05).

In order to establish a causal relationship between apoE genotype and neuronal staining for apoE.

**DISCUSSION**

The results presented here clearly demonstrate the presence of apoE-immunopositive neurons in human cerebral cortex. The most intense and widespread neuronal apoE IR was in NC and HPND sections, with little neuronal staining in AD sections where apoE was primarily localized to SPs and NFTs. We did not see significant glial apoE IR and apoE did not colocalize to GFAP immunopositive astrocytes. However, it is possible that low levels of apoE were not detected. Immunostaining identifies the steady state concentration of a protein but provides only suggestive evidence about its site of synthesis. It is possible that astrocytes produce and secrete apoE which is then internalized and concentrated in neurons. We are currently using in situ hybridization in these brain samples to localize apoE mRNA.

Our results are in apparent contrast to reports which found apoE primarily in astrocytes, with little apoE staining specific to neurons. A wide range of species display apoE IR in brain tissue, including rodents (2, 6) and non-human primates (3, 33, 34). In those species, the majority of apoE IR was in glial cells. In humans, reports of apoE immunostaining have been limited primarily to AD brain, where neurons harbor apoE immunoreactive NFTs (4, 16–18, 35), or to brains of patients with other neurological disorders (7, 36, 37). Investigations of apoE immunostaining in normal human brain have been very limited. Murakami and coworkers (5) immunostained a single normal control brain and demonstrated astrocytic apoE in numerous brain regions. In the hippocampus and temporal cortex from normal aged individuals, Rebeck and coworkers (4) report light apoE staining primarily of astrocytes. In a comparable study of the hippocampus, apoE IR was observed in astrocytes and blood vessels, as well as in neurons from 2 of 6 cases examined (7). In those studies, the absence of apoE-immunonegative neurons could have caused the faint staining of glial cells to appear more robust. While we also observed limited apoE staining of glial cells, it was dominated by intense, abundant apoE staining of neurons. In addition, we examined the frontal cortex, a region of the brain which may exhibit more extensive apoE neuronal staining than the hippocampus.

Detection of apoE in neurons may depend on several methodological considerations. Han and coworkers (36) observed the strongest apoE neuronal staining using a polyclonal antibody or a monoclonal antibody to the carboxyl terminus of apoE, suggesting that the conformation of apoE may be critical to the availability of a particular epitope. Although the conformation of apoE in vivo or in brain sections is unknown, it is possible that the structure of the protein is such that few epitopes are exposed. Thus, detection by immunostaining would depend on the nature of the antibody and the presentation of the apoE. The antibody used in the current study was rabbit polyclonal antiserum against human apoE. In immunoprecipitation and immunoblotting experiments, this antibody is highly selective in detecting apoE in whole plasma, as well as in cell lysates and media (38–40, unpublished observations). In the present study, the specificity of the apoE antiserum for immunohistochemistry was confirmed by preabsorbing the antibody with purified apoE prior to staining sections. This treatment effectively abolished all neuronal, SP, and NFT immunoreactivity associated with the apoE antiserum, as did staining with preimmune rabbit sera or secondary antibody alone.

Several studies have suggested that harsh methods of
tissue fixation and treatment reduce apoE immunostaining. Longer fixation times or high concentrations of formaldehyde or glutaraldehyde resulted in the specific loss of cellular apoE IR (36). In AD brain sections, formalin fixation and paraaffin-embedding of tissues abolished apoE IR in neuropil threads and intracellular NFTs and reduced staining in extracellular tangles and SPs (17). In addition, treatment of sections with formic acid or deparaffinizing agents decreased overall apoE IR (16, 36), while increasing apoE staining in plaques (36). Similarly, plaques were the last structure to lose apoE IR with antisera dilution (16). Recently, an investigation (41) using two different apoE antibodies on tissues processed identically to those in the current study has demonstrated neuronal apoE immunoreactivity similar to our results. These findings all suggest that milder conditions are necessary for the detection of cellular apoE, while extracellular staining is more robust. This could be due to differences in the vulnerability of apoE in the various deposits. Cellular apoE may be bound to the plasma membrane either by apoE receptors or through association with heparan sulfate proteoglycans (42, 43). Alternatively, apoE could be present intracellularly in secretory or endosomal vesicles. These apoE pools may be susceptible to chemical alteration during treatment with tissue fixatives or denaturants, either by stripping the protein from the membrane, disrupting the conformation of apoE, or otherwise removing the apoE epitope needed for antibody recognition. In contrast, the apoE in SPs may be protected from harsh conditions by being part of a dense, multi-protein complex. For the current study, mild conditions favorable for the detection of cellular apoE were used, which could account for the observed widespread neuronal apoE IR.

Previous hypotheses have attempted to explain the function of apoE isoforms in the pathological processes leading to AD. Rebeck and co-workers (4) proposed that apoE normally clears Aβ from the neuropil via upregulation by LRP on neurons. Because apoE3 and apoE4 bind LRP with equal affinity (44), they hypothesized that apoE4 was less efficient in clearing Aβ due to either an altered apoE-Aβ interaction or lower levels of apoE. Others propose that apoE4 lacks the ability to bind the microtubule-associated protein tau, leading to an accelerated rate of PHF formation and assembly into NFTs (37). These theories imply the presence of apoE in neurons. The neuronal distribution of apoE seen in the present study extends both of these models to include a role for apoE in normal brain function.

We have previously clarified the nature of the isoform-specific interaction between apoE and Aβ by demonstrating that native, but not purified, preparations of apoE3 bind to Aβ with greater avidity than apoE4 (39, 46). Thus, apoE3 may normally contribute to the clearance of soluble Aβ from the neuropil through binding to neuronal LRP, producing the observed apoE immunostaining of neurons. Reducing the concentration and residence time of extracellular Aβ could decrease the potential for amyloid deposition. Because apoE4 binds less effectively to Aβ, larger quantities of the peptide could remain available extracellularly for condensation into insoluble amyloid and contribute to AD pathogenesis. Indeed, the e4 allele correlates with higher amyloid plaque density in the brains of AD patients (4, 18). In this study, lower levels of neuronal apoE and LRP observed in AD brain sections may indicate that these tissues have a diminished capacity to clear Aβ through an intracellular pathway. In addition, apoE and LRP both localize to SPs, possibly facilitating the process of amyloid deposition.

Strittmatter and co-workers (45) have proposed that intraneuronal apoE3 binds and stabilizes microtubules to prevent the hyperphosphorylation of tau that results in the formation of NFTs. The genetic correlation of apoE4 with AD would therefore derive from the ineffectiveness of apoE4 in this function. The current finding that apoE colocalized to MAP-2 positive neurons in normal brain provides immunohistochemical support for biochemical evidence (37) that these 2 proteins interact within neurons. Furthermore, the diminished apoE, LRP, and MAP-2 neuronal staining in AD brain sections compared to controls may indicate that the normal interactions of these proteins are disrupted in AD.

These models propose a protective role of apoE3 in AD pathogenesis, a function that apoE4 lacks. However, several studies indicate that apoE4 directly catalyzes the formation of Aβ into amyloid fibrils (46–48), a process that could contribute to AD pathogenesis. Other data indicate that both apoE3 and apoE4 delay the onset of amyloid fibril formation, with apoE3 in its homodimeric form a more potent inhibitor of Aβ nucleation (49), again supporting the notion that apoE3 functions in a protective manner. Humans are the only species known to express apoE as different isoforms. With the exception of rabbits (Cys119), all other species have Arg at residues 112 and 158, comparable to human apoE4 at these sites. Moreover, the human apoE sequence diverges from other species at additional positions. Thus, it is possible that a protective role ascribed to apoE3 is unique to humans. The issue of whether it is the presence of apoE4 or the absence of apoE3 that contributes to the pathogenesis of AD is critical but remains unresolved.

In summary, we have used apoE immunostaining to identify neurons and senile plaques in the cerebral cortex of normal aged individuals and AD patients. These data clearly establish the consistent presence of apoE in neurons, and suggests apoE plays a role in these cells under non-disease conditions. This process appears to be altered by AD pathology, as the number of apoE-immunostained neurons was significantly reduced. These findings further support the genetic linkage between apoE and AD, and provide evidence to extend the biochemical hypotheses.

regarding apoE-Aβ or apoE-microtubule interactions to include a protective role for these complexes in neuronal function.

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