Journal of Neurochemistry
Lippincott-Raven Publishers, Philadelphia

β-Amyloid Peptides Increase the Binding and Internalization of Apolipoprotein E to Hippocampal Neurons

Uwe Beffert, Nicole Aumont, Doris Dea, *Suzanne Lussier-Cacan, *Jean Davignon, and Judes Poirier

*Douglas Hospital Research Centre, Department of Neurology and Neurosurgery, McGill University, and *Clinical Research Institute of Montreal, Montreal, Quebec, Canada*

Abstract: The frequency of the ε4 allele of apolipoprotein E (apoE) is increased in late-onset and sporadic forms of Alzheimer's disease (AD). ApoE also binds to β-amyloid (Aβ) and both proteins are found in AD plaques. To further investigate the potential interaction of apoE and Aβ in the pathogenesis of AD, we have determined the binding, internalization, and degradation of human apoE isoforms in the presence and absence of Aβ peptides to rat primary hippocampal neurons. We demonstrate that the lipophilic Aβ peptides, in particular $A\beta_{1-42}$, $A\beta_{1-40}$, and $A\beta_{25-35}$, increase significantly apoE-liposome binding to hippocampal neurons. For each Aβ peptide, the increase was significantly greater for the apoE4 isoform than for the apoE3 isoform. The most effective of the Aβ peptides to increase apoE binding, $A\beta_{25-35}$, was further shown to increase significantly the internalization of both apoE3- and apoE4-liposomes, without affecting apoE degradation. Conversely, $A\beta_{1-40}$ uptake by hippocampal neurons was shown to be increased in the presence of apoE-liposomes, more so in the presence of the apoE4 than the apoE3 isoform. These results provide evidence that Aβ peptides interact directly with apoE lipoproteins, which may then be transported together into neuronal cells through apoE receptors. **Key Words:** Alzheimer's disease—Apolipoprotein E—β-Amyloid peptide—Hippocampal neurons—Apolipoprotein E binding—Liposomes—Apolipoprotein E3 and E4 isoforms.
J. Neurochem. **70,** 1458–1466 (1998).

Apolipoprotein E (apoE) is a lipid-binding, 34-kDa, 299-amino acid protein involved in cholesterol and phospholipid transport and metabolism. ApoE mediates the uptake of lipid complexes by acting as a ligand for several receptors including the low-density lipoprotein (LDL) receptor (Mahley, 1988), the LDL receptor-related protein (LRP) (Beisiegel et al., 1989), the very-low-density lipoprotein (VLDL) receptor (Takahashi et al., 1992), and also possibly through the newly discovered apoE receptor 2 (apoER2) (Kim et al., 1996). The LDL receptor pathway consists of cell surface binding of apoE-containing lipoproteins followed by internalization and degradation of the apoE complex by a lysosomal pathway (Goldstein et al., 1983). The major sites of apoE synthesis are the liver, brain, spleen, lung, adrenal, ovary, kidney, and muscle (Elshourbagy et al., 1985). In the brain, apoE mRNA is present mostly in astrocytes (Poirier et al., 1991), and apoE is found in the CSF as a component of lipoproteins and lipid complexes (Boyles et al., 1985; Pitas et al., 1987*a,b*). Several apoE receptors have been identified in the brain, including the LDL receptor (Pitas et al., 1987*b*; Swanson et al., 1988), LRP (Wolf et al., 1992; Rebeck et al., 1993), VLDL receptor (Gafvels et al., 1994; Christie et al., 1996), and apoER2 (Kim et al., 1996).

The structural gene for apoE is polymorphic, leading to three major isoforms, designated apoE2, apoE3, and apoE4, which are derived from the ε2, ε3, and ε4 alleles, respectively (Davignon et al., 1988). Among the major apoE isoforms, the frequency of the apoE ε4 allele is increased significantly in sporadic and late-onset familial cases of Alzheimer's disease (AD) compared with the general population (Corder et al., 1993; Poirier et al., 1993; Rebeck et al., 1993; Saunders et al., 1993). The physiological role of apoE in the pathogenesis of AD is presently unknown; however, growing evidence points to a key role for apoE in synaptic plasticity (Poirier, 1994; Poirier et al., 1995; Arendt et al., 1997). Another theory involves an interaction of apoE with the amyloidogenic fragments of the amyloid precursor protein (APP). Recent evidence suggests

Received September 18, 1997; revised manuscript received November 17, 1997; accepted November 20, 1997.

Address correspondence and reprint requests to Dr. J. Poirier at McGill Centre for Studies in Aging, 6825 LaSalle Boulevard, Verdun, QC, Canada H4H 1R3.

Abbreviations used: Aβ, β-amyloid; AD, Alzheimer's disease; apoE, apolipoprotein E; apoER2, apoE receptor 2; APP, amyloid precursor protein; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; HDL, high-density lipoprotein; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; LRP, LDL receptor-related protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; VLDL, very-low-density lipoprotein.

apoE and β-amyloid (Aβ) peptides form stable sodium dodecyl sulfate (SDS)-resistant complexes, indicative of strong intermolecular binding (Strittmatter et al., 1993; LaDu et al., 1994, 1995; Yang et al., 1997). ApoE has been shown to reduce polymerization of Aβ in vitro (Evans et al., 1995) as well as reduce Aβ toxicity in neuronal cell cultures (Whitson et al., 1994; Puttfarcken et al., 1997). By using autopsied AD brain tissue, apoE has also been localized to extracellular senile plaques, intracellular neurofibrillary tangles, and vascular amyloid, the neuropathological lesions characteristic of AD (Namba et al., 1991). Strong associations have been found between the apoE4 allele and increased vascular and parenchymal Aβ deposition (Schmechel et al., 1993; Beffert and Poirier, 1996). Collectively, these data have led to the hypothesis that apoE and Aβ interact by some physiological mechanism that underlies the pathogenesis of AD.

In the present study, we have sought to further define the functional role of apoE and the potential interaction with Aβ. We have characterized the binding, internalization, and degradation of the two most common apoE isoforms on rat primary hippocampal neurons in the presence and the absence of Aβ peptides. We demonstrate that apoE-liposome binding and internalization are increased by Aβ peptides in an isoform-specific manner and, conversely, that neuronal Aβ uptake can be increased by apoE-liposomes. These data further establish a physiological link between apoE, Aβ, and AD.

MATERIALS AND METHODS

Materials

Various Aβ peptides including $A\beta_{1-40}$, $A\beta_{1-42}$, $A\beta_{1-28}$, $A\beta_{25-35}$, and $A\beta_{40-1}$ were purchased from Bachem (Torrance, CA, U.S.A.). The scrambled peptide $A\beta_{25-35}$ was a kind gift of P. Gaudreau (Notre-Dame Hospital Research Centre, Montreal, Quebec, Canada). Materials used for cell culture were obtained from GibcoBRL (Rockville, MD, U.S.A.). Bolton–Hunter reagent was purchased from Du Pont–NEN (Boston, MA, U.S.A.). Unless otherwise stated, all other chemicals were obtained from Sigma (Oakville, Ontario, Canada).

ApoE purification

Human apoE was purified from the plasma of pregenotyped apoE-homozygous individuals as previously described (Guillaume et al., 1996). In brief, 10 μ*M* phenylmethylsulfonyl fluoride, 0.3 m*M* EDTA, and 3 m*M* sodium azide were added to all sera, which were then ultracentrifuged at 8.5 × 10^4 *g* for 30 min at 16°C and then twice at 1.8 × 10^6 *g*-h to isolate and wash the VLDL. An equal volume of isopropanol was added to a 1.5 mg/ml VLDL solution during vigorous agitation, followed by centrifugation at 10,000 *g* for 10 min. The isopropanol was evaporated under a jet of nitrogen, and acetone (1.2 times the volume) was added to the remaining solution, which was centrifuged at 20,000 *g* for 20 min. The pellet containing apoE was resuspended in NaCl (150 m*M*) and the acetone extraction was repeated. Solvent-extracted apolipoproteins were delipidated by extracting twice with chloroform/methanol (2:1), each time followed by centrifugation at 20,000 *g* for 20 min. Delipidated proteins were dissolved in Tris-HCl buffer (10 m*M*; pH 7.4) containing NaCl (150 m*M*), EDTA (0.3 m*M*), and urea (5.0 *M*). Solutions were then centrifuged at 10,000 *g* for 10 min and then dialysed against the same buffer containing no urea.

ApoE-liposome construction

ApoE-containing liposomes were prepared according to the method of Matz and Jonas (1982) with the following conditions: 100 mol of phosphatidylcholine/100 mol of cholate/25 mol of cholesterol/mol of apoE. The mixtures were incubated for 12 h at 4°C and were dialysed for 48 h with four changes of Tris-HCl (10 m*M*) buffer, pH 7.4, containing NaCl (150 m*M*) and EDTA (0.3 m*M*). ApoE-liposomes were isolated via centrifugation at 3 × 10^6 *g*-h at a density of 1.125 g/ml, and the isolated fraction was dialysed against a NaCl (150 m*M*) solution, pH 7.5, containing 0.3 m*M* EDTA and 3 m*M* sodium azide.

ApoE radioiodination

ApoE-liposomes were labeled according to the Bolton–Hunter procedure (Innerarity et al., 1979). Human apoE (100–200 μg) in 100 μl of potassium phosphate buffer (pH 7.5) was added to 1 mCi of dried Bolton–Hunter reagent. The reaction was left on ice for 4 h at 4°C with occasional gentle agitation. The labeling reaction was terminated with 200 μl of glycine (1 mg/ml in potassium phosphate buffer). The radiolabeled apoE was then separated, using a preequilibrated (0.1% gelatin in phosphate buffer) Sephadex G-25 (Pharmacia) column, and the protein fractions were pooled and collected. Protein–lipid labeling ratios were monitored routinely and found to be ~95:5 for protein/lipid.

Hippocampal neurons

Primary hippocampal neuronal cells were obtained from the hippocampus of embryonic (E18–19) Sprague–Dawley rats (Charles River). All procedures were performed in accordance with the Canadian Council on Animal Care Guidelines and the Society for Neuroscience Policy on the Use of Animals in Research. Neurons were plated at a density of 5 × 10^5 cells/16-mm^2 well in poly-D-lysine-coated tissue culture dishes and maintained in a serum-free supplemented growth medium (GIBCO B27). On the day after plating, the cell culture medium was replaced with fresh medium. Experiments were performed after 6 days in culture, at which time the pyramidal neurons are fully differentiated (Mattson et al., 1991). By using these procedures, contamination by glial and other cell types is limited to <2.5% of all cells.

ApoE-liposome and Aβ preincubation

Aβ peptides were dissolved in 3% dimethyl sulfoxide in double-distilled H_2O at a stock concentration of 1 mg/ml. Unused aliquots were stored at −80°C until further use. ^{125}I-labeled apoE-liposomes (see Figs. 1–3) and unlabeled apoE-liposomes (see Fig. 4) were then preincubated with the indicated Aβ peptides for 2 h at 37°C, with the preincubation concentrations proportional to the final concentrations indicated. Treated neurons were monitored routinely for toxicity by using either lactate dehydrogenase (LDH) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.

Binding, internalization, and degradation

Binding assays of ^{125}I-labeled apoE-liposomes were measured as described by Innerarity et al. (1986). For 4°C studies

(see Figs. 1 and 2), cells were chilled on ice with ice-cold Dulbecco's modified Eagle medium (DMEM) with 0.2% bovine serum albumin (BSA). ApoE-liposome binding was determined by incubating the indicated concentrations of ligand with or without Aβ peptide for 4 h at 4°C in the presence and absence of a 500-fold excess of unlabeled LDL. Neurons were then washed thoroughly with ice-cold phosphate-buffered saline (PBS) buffer with 0.2% BSA and then again with PBS. Cells were then detached with 0.1 *M* NaOH and the amount of radioactivity determined. For 37°C studies, neurons were incubated with ligand as above for 4 h. The incubation medium was then removed and the degradation products determined from the trichloroacetic acid-soluble supernatant as previously described (Goldstein et al., 1983). Cells were then washed on ice with PBS as above and then incubated with ice-cold 25 m*M* sodium suramin for 1 h on a rotary shaker. The released fraction of radioactivity was used to determine the bound fraction. Cells were then rinsed with PBS and detached with 0.1 *M* NaOH to determine the internalized fraction. An aliquot of this solution was used to determine the protein concentration per well. Results were expressed as nanograms of ^{125}I-apoE per milligram of total protein. K_D data are expressed as mean ± SEM values. The data shown are the result of triplicate experiments, using different batches of cells unless otherwise noted.

Western blotting experiments

Unlabeled apoE and Aβ were preincubated for 2 h at 37°C as above. The mixture was then diluted to concentrations of 1 μg/ml for both apoE and Aβ. The cells were incubated with this mixture in DMEM for a period of 24 h. The cells were then harvested from the Petri dishes and sonicated briefly to disrupt the membranes. Samples were pelleted, aliquoted, and stored at −80°C for protein assay. By using the method of Laemmli (1970), cell homogenates (25 μg of total protein) were mixed with 5× reducing sample buffer, boiled for 1 min, and run on Novex 12% Tris-glycine gels for apoE or 10–20% tricine gels for Aβ (Helixx Technologies, Canada) for SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes (Amersham) and probed with antibodies to apoE or Aβ. For apoE detection, membranes were probed with a polyclonal goat anti-human apoE antibody (IIC; Murrieta, CA, U.S.A.) diluted 1:2,000. Aβ detection was performed by using monoclonal antibody 4G8 recognizing Aβ peptides 17–24 (1:500; kindly provided by M. R. Emmerling). All membranes were visualized by using Amersham's enhanced chemiluminescence kit and exposed to Kodak XAR5 film.

Statistics

Binding parameters were determined by using GraphPad Prism 2.0 software (San Diego, CA, U.S.A.). A one-way ANOVA or Student's *t* test was used to test the significance of changes observed under different conditions. Quantification of autoradiographic signals was performed by using the MCID image-analysis system (St. Catharines, Ontario, Canada) equipped with one-dimensional gel-analysis software.

RESULTS

Saturation binding of apoE to hippocampal neurons

To demonstrate apoE function in the CNS, we decided to establish a model that mimics as closely as possible the conditions found in this environment. Liposome complexes of human apoE were used, primarily because it has been shown that total delipidation of apoE-containing lipoproteins abolishes the ability of apoE to bind to its receptors (Innerarity et al., 1979). Second, apoE has been shown to be present in human CSF as high-density lipoprotein (HDL)-like particles that are comparable in size with the liposomes used here (Pitas et al., 1987*b*). Furthermore, the CSF concentration of apoE in rats is similar to that found in humans, ~450 μg/dl (Chiba et al., 1991). Binding of human apoE-liposome complexes to rat hippocampal neurons therefore provides an excellent model for investigating the function of apoE in the CNS.

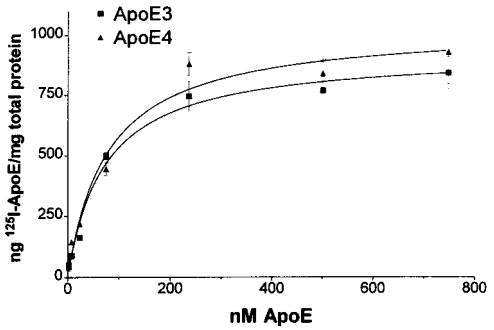

FIG. 1. Saturation binding curves of ^{125}I-apoE3- and ^{125}I-apoE4-liposomes at 4°C for 4 h to primary rat hippocampal neurons. Specific binding as determined from the difference between total binding in the presence and the absence of 500-fold LDL is plotted against increasing concentrations of apoE-liposomes. K_D and B_{max} were determined by nonlinear regression of the saturation binding curve.

Human apoE3- and apoE4-liposomes bound avidly and comparably with rat hippocampal neurons at 4°C, yielding a typical saturation profile (Fig. 1). Nonlinear regression analysis revealed an average K_D of 72.7 ± 13.6 n*M* for apoE3 and 78.5 ± 18.8 n*M* for apoE4. Maximum binding was determined yielding B_{max} = 921.5 ± 43.0 and 1,031.0 ± 62.9 ng ^{125}I-apoE/mg of total protein for apoE3- and apoE4-liposomes, respectively. Nonspecific binding was determined in the presence of an excess of 500-fold unlabeled LDL. A 100-fold excess of unlabeled human apoE3-liposomes and apoE4-liposomes was equally effective as a 500-fold excess of human LDL in displacing the binding of the labeled ligands, with 500-fold human VLDL displaying slightly less efficacy (Table 1). Liposomes containing no native apolipoprotein were ineffective at displacing either iodinated ligand.

ApoE binding with preincubated Aβ

Iodinated apoE and Aβ were preincubated for 2 h at 37°C to maximize potential protein–protein interactions. The preincubation period of 2 h used here was previously shown to demonstrate maximal apoE isoform-specific differences in binding to Aβ (Strittmatter et al., 1993). The mixture was then diluted into the

TABLE 1. *Displacement of human apoE-liposome complexes to hippocampal neurons*

Lipoprotein	Concentration (μg/ml)	% of displaced ^{125}I-apoE3	% of displaced ^{125}I-apoE4
Human apoE3	100	78	75
Human apoE4	100	79	74
LDL	500	70	68
VLDL	500	64	65
Liposomes only[a]		0	0

[a] The concentration of liposomes was 17.5 μg of liposomes/ml of media. This is the same concentration of liposomes present in the apoE-liposomes at a concentration of 100 μg/ml.

cell culture media used for the binding experiment. Binding experiments were then performed at 4°C as described in Materials and Methods. Figure 2A demonstrates that $A\beta_{25-35}$ was capable of increasing significantly the binding of both apoE3- and apoE4-liposomes to rat hippocampal neurons at concentrations as low as 10^{-9} *M* ($p < 0.0001$). The increase in binding was maximal at 10^{-6} *M* $A\beta_{25-35}$ for both apoE3- and apoE4-liposomes. Furthermore, apoE4-liposome binding was consistently higher than apoE3-liposome binding at equal concentrations of $A\beta_{25-35}$ ($F = 21.57$; *df* 1,108; $p < 0.0001$). In contrast, two control peptides, scrambled $A\beta_{25-35}$ and $A\beta_{40-1}$, were ineffective at increasing apoE3- or apoE4-liposome binding to hippocampal neurons (Fig. 2B). Figure 2C and D demonstrates the binding of apoE3- and apoE4-liposomes in the presence of increasing concentrations of $A\beta_{1-28}$, $A\beta_{1-40}$, and $A\beta_{1-42}$. $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides displayed similar efficacy at increasing the binding of both apoE3- and apoE4-liposomes to hippocampal neurons. Both $A\beta_{1-40}$ and $A\beta_{1-42}$ increased apoE4-liposome binding significantly and comparably more than apoE3-liposome binding at equimolar concentrations ($F = 17.99$; *df* 1,108; $p < 0.0001$ for $A\beta_{1-40}$; $F = 49.80$; *df* 1,108; $p < 0.0001$ for $A\beta_{1-42}$). $A\beta_{1-28}$ peptide did not increase the binding of either apoE3- or apoE4-liposomes compared with control values ($p > 0.05$).

The Aβ peptides used here in their aggregated forms are toxic to many cell types including neurons (Pike et al., 1993; Takadera et al., 1993). However, apoE has also been shown to inhibit Aβ fibril formation

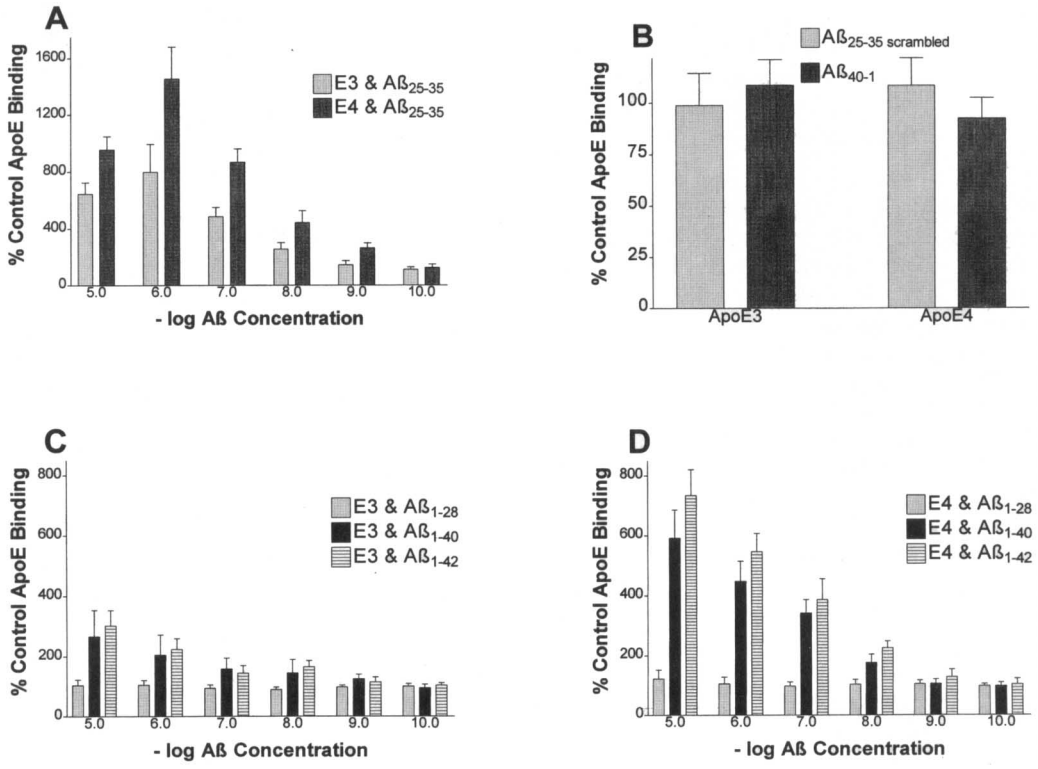

FIG. 2. Binding of ^{125}I-apoE3- and ^{125}I-apoE4-liposomes at 4°C to primary rat hippocampal neurons in the presence of Aβ peptides. ApoE-liposomes and Aβ peptides were preincubated for 2 h at 37°C and then diluted into fresh incubation medium for 4 h for binding to neurons. Data are presented as percentage of control apoE binding versus Aβ concentration where the binding of apoE3- and apoE4-liposomes was established as 100%. **A:** ^{125}I-apoE3- and ^{125}I-apoE4-liposomes (1 μg/ml or 29 n*M*) with increasing concentrations of $A\beta_{25-35}$. **B:** ^{125}I-apoE3- and ^{125}I-apoE4-liposomes with 10^{-6} *M* scrambled $A\beta_{25-35}$ or $A\beta_{40-1}$. **C** and **D:** ^{125}I-apoE3- and ^{125}I-apoE4-liposomes (1 μg/ml) with increasing concentrations of $A\beta_{1-42}$, $A\beta_{1-40}$, and $A\beta_{1-28}$, respectively. Scales of C and D were maintained to facilitate comparison of apoE isoforms.

(Evans et al., 1995) as well as decrease the toxicity induced by Aβ peptides in rat hippocampal neuronal cultures (Puttfarcken et al., 1997). We routinely verified neuronal toxicity with both MTT and LDH assays and found that conditions incubated with combinations of apoE and Aβ displayed no variations from control, untreated cell cultures (data not shown).

Binding, internalization, and degradation of apoE isoforms with and without Aβ_{25-35}

Because the Aβ_{25-35} fragment was found to be the most potent at increasing the binding of apoE to hippocampal neurons, it was considered appropriate to continue with this peptide for further analysis of internalization and degradation. It is noteworthy that the Aβ_{25-35} peptide is also the proposed biologically active fragment of Aβ (Yankner et al., 1990). Figure 3 demonstrates the increase in 37°C binding and internalization, using a range of apoE3- and apoE4-liposome concentrations in the presence of a fixed concentration of Aβ_{25-35} (1 μg/ml). Nonlinear regression was used to fit data points to the curves. Both apoE-liposome preparations demonstrated significant increases in binding (Fig. 3A and B) and internalization (Fig. 3C and D) due to the presence of Aβ_{25-35} ($p < 0.0001$, in each case). At equimolar concentrations of apoE, Aβ_{25-35} increased the amount of binding and internalization of apoE4-liposomes more than apoE3-liposomes ($p < 0.0001$). The amount of degradation measured was not affected by the presence of Aβ_{25-35}; however, apoE3-liposome degradation was found to be significantly higher than apoE4-liposome degradation at equimolar concentrations ($p < 0.0001$; Fig. 3E and F).

ApoE and Aβ uptake by immunoblot

To determine if the increase in apoE binding and internalization to neurons also affected Aβ levels, parallel experiments were run to determine apoE and Aβ uptake by measuring immunoreactive levels of apoE and Aβ. Unlabeled apoE3- and apoE4-liposomes were preincubated with Aβ peptides and then incubated in the cell culture medium for 24 h at 37°C. Immunoblots for apoE (Fig. 4A and B) yielded results similar to those for apoE3- and apoE4-liposome binding and internalization. Aβ_{25-35} was the most potent of the Aβ peptides tested, at increasing the amount of apoE immunoreactivity measured from neuronal cell homogenates. Aβ_{25-35} increased significantly both apoE3 and apoE4 levels as determined by immunoblot compared with conditions incubated with apoE3 or apoE4 alone ($p < 0.01$). Aβ_{1-40} also increased apoE immunoreactive levels but was significant only for the apoE4 isoform ($p < 0.05$). Control peptide Aβ_{1-28} did not increase immunoreactive apoE levels significantly ($p > 0.05$).

Because the apoE-immunoreactive levels measured were complementary to those obtained by the classic binding and internalization study, it was reasonable to assume that Aβ-immunoreactive levels may be interpreted in a similar fashion, i.e., that the immunoreactive Aβ levels represent the binding and internalization (or uptake) of Aβ by the hippocampal neurons. Immunoblots for Aβ indicated that significantly higher levels of Aβ_{1-40} were detectable in hippocampal neurons in the presence of apoE4-liposomes in comparison with apoE3-liposomes (Fig. 4C; $p < 0.01$). The antibodies used were not able to detect the Aβ_{25-35} peptide.

DISCUSSION

The central findings of this study indicate that Aβ peptides increase effectively the binding and internalization of apoE-liposomes to hippocampal neurons. The increase in binding was greater for the apoE4 isoform compared with the apoE3 isoform for each of the Aβ peptides tested. These results are consistent with several lines of evidence linking apoE and Aβ. First, apoE4 obtained from purified delipidated plasma (as used here before recombination with liposomes) has been shown to bind more avidly to Aβ than apoE3 (Strittmatter et al., 1993). More recent studies have challenged this finding (LaDu et al., 1994, 1995; Zhou et al., 1996; Yang et al., 1997); however, it should be noted that the interaction of apoE and Aβ in the presence of lipids has not yet been thoroughly evaluated, which may be critical because apoE is thought to undergo a conformational change when associated with lipid (Lund-Katz et al., 1993; Weisgraber, 1994). Second, brain tissue from AD patients homozygous for the apoE ϵ4 allele display significantly more Aβ plaques than their ϵ3 counterparts, perhaps reflective of a direct pathogenetic role for apoE in amyloid deposition (Rebeck et al., 1993; Schmechel et al., 1993; Beffert and Poirier, 1996). At the present time, it is difficult to conclude precisely what this increase in apoE binding represents, and furthermore, how this may relate directly to the pathology of AD. The exact roles of both apoE and Aβ in the brain are yet to be defined; however, if the primary role of apoE in the brain is to aid in neuronal plasticity (Poirier, 1994), an increase in binding may be a response to increased neuronal damage/stress brought about by sublethal doses of Aβ.

An alternate possibility is that apoE may serve multiple roles in the CNS, one of which may be to clear Aβ from the extracellular space through its receptors, which may be reflected by increased apoE binding activity. To this end, we have demonstrated that apoE-liposomes increase the uptake of Aβ_{1-40} in hippocampal neurons as detected by western blotting after a 24-h incubation. Although this method does not distinguish binding and internalization, it does indicate that steady-state, bound/internalized levels of Aβ are affected by the different apoE-liposome preparations used here. The removal of Aβ from the extracellular space through apoE receptors, after complex formation with apoE-liposomes or other lipoproteins, may therefore prevent the formation of Aβ fibrils and provide protection against toxicity. This hypothesis is consistent with

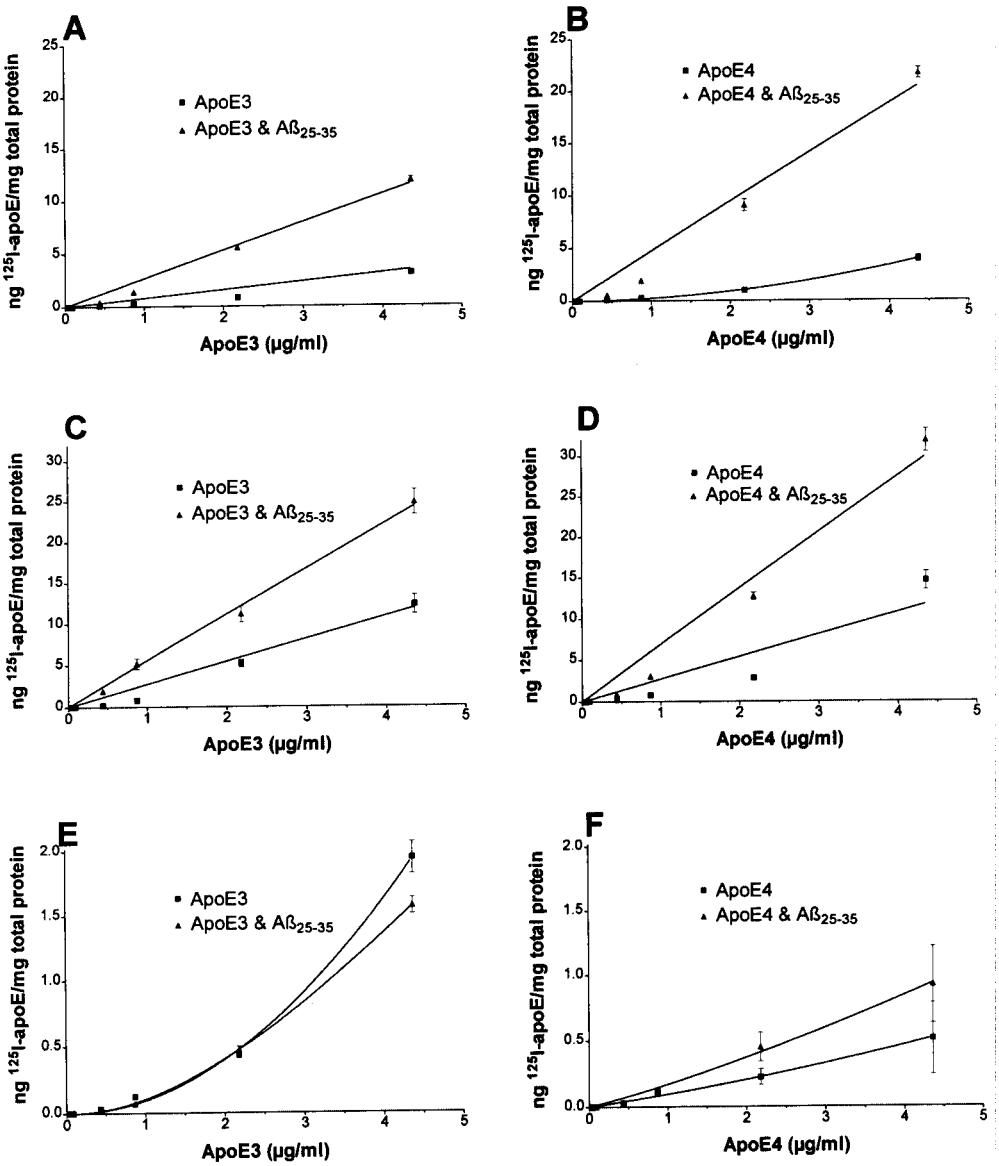

FIG. 3. Binding (**A** and **B**), internalization (**C** and **D**), and degradation (**E** and **F**) of ^{125}I-apoE3-liposomes (A, C, and E) and ^{125}I-apoE4-liposomes (B, D, and F) after 4-h incubation at 37°C to primary rat hippocampal neurons in the presence of 1 μM Aβ_{25-35} peptide. The bound and internalized fractions were determined from the fractions that were sensitive or resistant to release by treatment with 25 mM sodium suramin. Degradation was determined from the trichloroacetic acid-nonprecipitable supernatant from the incubation media.

several lines of evidence. First, CSF Aβ_{1-42} levels have been found to be lowered in AD compared with controls, perhaps due to increased clearance (Motter et al., 1995; Ida et al., 1996). Second, microglial cells have been shown to internalize aggregates of Aβ peptides through a scavenger receptor, whose ligands include acetylated and oxidized LDL (Paresce et al., 1996). Finally, recent work has shown that the secreted form of APP can be internalized and degraded through LRP, one of the central apoE receptors in the brain (Kounnas et al., 1995). Based on these observations, we hypothesize that Aβ peptides interact directly with apoE lipoproteins, forming a complex that is bound and internalized into neuronal cells through apoE receptors. Together, these results further establish a functional relationship between apoE and Aβ, which strengthens their combined role in the pathogenesis of AD.

The most common amyloidogenic fragments of AD neuritic plaques include Aβ_{1-42} and Aβ_{1-40} (Selkoe, 1994). We have found that Aβ_{1-42}, Aβ_{1-40}, and Aβ_{25-35} are all effective at significantly increasing apoE3- and apoE4-liposome binding to hippocampal neurons. Each of these fragments is obtained from the lipophilic domain of the APP molecule (Selkoe, 1994). This is consistent with studies showing that 5% of Aβ added to plasma is bound to HDL, LDL, and VLDL particles (Biere et al.,

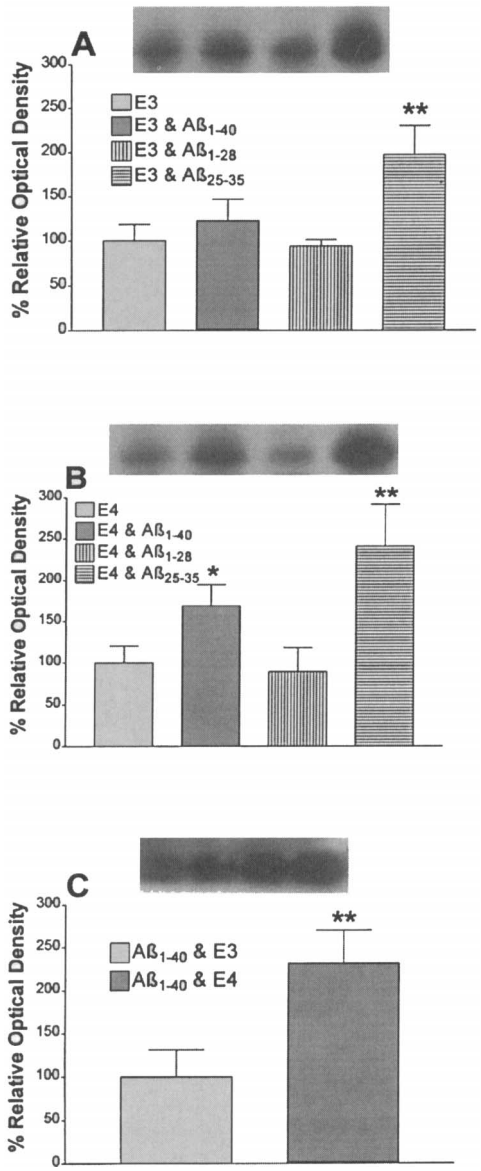

FIG. 4. Relative immunoblot levels of apoE3 (**A**), apoE4 (**B**), and $A\beta_{1-40}$ (**C**) obtained from neuronal cell homogenates after incubation with the indicated combinations of apoE and $A\beta$ at 1 μg/ml each for 24 h at 37°C. For A and B, the **insets** demonstrate one representative immunoreactive band for each condition. In C, the **inset** demonstrates duplicate bands for $A\beta_{1-40}$ in the presence of apoE3- and apoE4-liposomes. Quantified signals were normalized for background (0%) and the first signal of each figure (100%) and each represents the mean of triplicate values from two separate cultures. The apoE-immunoreactive band was obtained at ~37 kDa and the $A\beta$ band at ~4 kDa. $^*p < 0.05$; $^{**}p < 0.01$, compared with the first column on each plot.

1996). A growing body of evidence now implicates cholesterol and lipids in $A\beta$ regulation in the CNS (Hartmann et al., 1994; Bodovitz and Klein, 1996; Kim and Suh, 1996; Koudinov et al., 1996; Koudinova et al., 1996). In other control binding experiments, we demonstrate that $A\beta$ peptides including the lipophobic $A\beta_{1-28}$ fragment, scrambled $A\beta_{25-35}$, and the reversed sequence $A\beta_{40-1}$ had no effect on apoE-liposome binding. Together, these results indicate that the $A\beta$ peptide has a lipophilic sequence-specific region that interacts with apoE-liposomes to increase apoE binding to hippocampal neurons.

One of the proposed functional roles for apoE in the brain is to aid in the process of neuronal plasticity by mediating the cellular uptake of lipoprotein particles through receptors of the LDL receptor family (Poirier, 1994). We have demonstrated that both plasma human apoE3 and apoE4 isoforms bind comparably with rat primary hippocampal neurons in a specific and saturable process. These data are consistent with the comparable LDL receptor binding of apoE3- and apoE4-lipoprotein complexes to several other cell types (Weisgraber et al., 1982; Cassel et al., 1984). With regard to neuronal cells, previous work by Ignatius et al. (1987) demonstrated that apoE-containing lipid particles and lipoproteins were internalized by neurites and growth cones of pheochromocytoma cells. They implied that much of the internalization occurred through LDL receptors located in the growth cones and cell body of these cells. More recently, Bellosta et al. (1995) have demonstrated that uptake of β-VLDL occurred to a similar extent in both apoE3- and apoE4-expressing murine neuroblastoma cells. Their data show that the LRP is primarily responsible for the uptake of lipid particles into ganglionic neuronal cells. We have further shown that the degradation of apoE3-liposome complexes is greater than that of apoE4-liposome complexes to hippocampal neurons. Differences in apoE isoform-specific binding, internalization, and degradation through neuronal LRP and/or LDL receptors may be responsible for the differential neurite outgrowth and development reported in several studies (Bellosta et al., 1995; Narita et al., 1997). To date, a full characterization of all apoE receptors including the LDL receptor, LRP, VLDL receptor, and apoER2 on rat neuronal cells has not been performed. Combined, these findings confirm that apoE-liposome complexes are bound, internalized, and degraded through LDL receptors and/or LRP and that this interaction may be an important functional role for apoE in the brain.

To summarize, we have shown that $A\beta$ peptides increase significantly the binding of apoE-liposomes to hippocampal neurons, with apoE4 binding increased to a greater extent than apoE3. The lipophilic fragments of $A\beta$ were found to be responsible for mediating the increase in binding. The different binding properties of apoE4 versus apoE3 to hippocampal neurons in the presence of $A\beta$ may provide mechanistic understanding of the genetic association of apoE to AD.

Acknowledgment: This study was supported by grants from the Fonds de la Recherche en Santé du Québec (FRSQ), the Alzheimer Society of Canada and the Medical Research Council of Canada (MRCC) to J.P. U.B. is a recipient of studentships from the Fonds pour la Formation de Cher-

cheurs et l'Aide à la Recherche (FCAR) and from the Alzheimer Society of Montreal. We thank Satyabrata Kar and Melanie Reuben for helpful comments.

REFERENCES

Arendt T., Schindler C., Bruckner M. K., Eschrich K., Bigl V., Zedlick D., and Marcova L. (1997) Plastic neuronal remodeling is impaired in patients with Alzheimer's disease carrying apolipoprotein ε4 allele. *J. Neurosci.* **17,** 516–529.

Beffert U. and Poirier J. (1996) Apolipoprotein E, plaques, tangles and cholinergic dysfunction in Alzheimer's disease. *Ann. NY Acad. Sci.* **777,** 166–174.

Beisiegel U., Weber W., Ihrke G., Herz J., and Stanley K. K. (1989) The LDL-receptor-related protein, LRP, is an apolipoprotein E-binding protein. *Nature* **341,** 162–164.

Bellosta S., Nathan B. P., Orth M., Dong L. M., Mahley R. W., and Pitas R. E. (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.

Biere A. L., Ostaszewski B., Stimson E. R., Hyman B. T., Maggio J. E., and Selkoe D. J. (1996) Amyloid beta-peptide is transported on lipoproteins and albumin in human plasma. *J. Biol. Chem.* **271,** 32916–32922.

Bodovitz S. and Klein W. L. (1996) Cholesterol modulates alpha-secretase cleavage of amyloid precursor protein. *J. Biol. Chem.* **271,** 4436–4440.

Boyles J. K., Pitas R. E., Wilson E., Mahley R. W., and Taylor J. M. (1985) Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system. *J. Clin. Invest.* **76,** 1501–1513.

Cassel D. L., Phillips M. C., Rostron P., Rothblat G. H., and Utermann G. (1984) The conformation of apolipoprotein E isoforms in phospholipid complexes and their interaction with human Hep G2 cells. *Atherosclerosis* **52,** 203–218.

Chiba H., Mitamura T., Fujisawa S., Ogata A., Aimoto Y., Tashiro K., and Kobayashi K. (1991) Apolipoproteins in rat cerebrospinal fluid: a comparison with plasma lipoprotein metabolism and effect of aging. *Neurosci. Lett.* **133,** 207–210.

Christie R. H., Chung H., Rebeck G. W., Strickland D., and Hyman B. T. (1996) Expression of the very low-density lipoprotein receptor (VLDL-r), an apolipoprotein-E receptor, in the central nervous system and in Alzheimer's disease. *J. Neuropathol. Exp. Neurol.* **55,** 491–498.

Corder E. H., Saunders A. M., Strittmatter W. J., Schmechel D. E., Gaskell P. C., Small G. W., Roses A. D., Haines J. L., and Pericak-Vance M. A. (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* **261,** 921–923.

Davignon J., Gregg R. E., and Sing C. F. (1988) Apolipoprotein E polymorphism and atherosclerosis. *Arteriosclerosis* **8,** 1–21.

Elshourbagy N. A., Liao W. S., Mahley R. W., and Taylor J. M. (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.

Evans K. C., Berger E. P., Cho C. G., Weisgraber K. H., and Lansbury P. T. Jr. (1995) Apolipoprotein E is a kinetic but not a thermodynamic inhibitor of amyloid formation: implications for the pathogenesis and treatment of Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **92,** 763–767.

Gafvels M. E., Paavola L. G., Boyd C. O., Nolan P. M., Wittmaack F., Chawla A., Lazar M. A., Bucan M., Angelin B. O., and Strauss J. F. 3rd (1994) Cloning of a complementary deoxyribonucleic acid encoding the murine homolog of the very low density lipoprotein/apolipoprotein-E receptor: expression pattern and assignment of the gene to mouse chromosome 19. *Endocrinology* **135,** 387–394.

Goldstein J. L., Basu S. K., and Brown M. S. (1983) Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol.* **98,** 241–260.

Guillaume D., Bertrand P., Dea D., Davignon J., and Poirier J. (1996) Apolipoprotein E and low-density lipoprotein binding and internalization in primary cultures of rat astrocytes: isoform-specific alterations. *J. Neurochem.* **66,** 2410–2418.

Hartmann H., Eckert A., and Muller W. E. (1994) Apolipoprotein E and cholesterol affect neuronal calcium signalling: the possible relationship to beta-amyloid neurotoxicity. *Biochem. Biophys. Res. Commun.* **200,** 1185–1192.

Ida N., Hartmann T., Pantel J., Schroder J., Zerfass R., Forstl H., Sandbrink R., Masters C. L., and Beyreuther K. (1996) Analysis of heterogeneous A4 peptides in human cerebrospinal fluid and blood by a newly developed sensitive western blot assay. *J. Biol. Chem.* **271,** 22908–22914.

Ignatius M. J., Shooter E. M., Pitas R. E., and Mahley R. W. (1987) Lipoprotein uptake by neuronal growth cones in vitro. *Science* **236,** 959–962.

Innerarity T. L., Pitas R. E., and Mahley R. W. (1979) Binding of arginine-rich (E) apoprotein after recombination with phospholipid vesicles to the low density lipoprotein receptors of fibroblasts. *J. Biol. Chem.* **254,** 4186–4190.

Innerarity T. L., Pitas R. E., and Mahley R. W. (1986) Lipoprotein-receptor interactions. *Methods Enzymol.* **129,** 542–565.

Kim D. H., Iijima H., Goto K., Sakai J., Ishii H., Kim H. J., Suzuki H., Kondo H., Saeki S., and Yamamoto T. (1996) Human apolipoprotein E receptor 2. A novel lipoprotein receptor of the low density lipoprotein receptor family predominantly expressed in brain. *J. Biol. Chem.* **271,** 8373–8380.

Kim S.-H. and Suh Y.-H. (1996) Neurotoxicity of a carboxyl-terminal fragment of the Alzheimer's amyloid precursor protein. *J. Neurochem.* **67,** 1172–1182.

Koudinov A. R., Koudinova N. V., and Berezov T. T. (1996) Alzheimer's peptides Aβ-1-40 and Aβ-1-28 inhibit the plasma cholesterol esterification rate. *Biochem. Mol. Biol. Int.* **38,** 747–752.

Koudinova N. V., Berezov T. T., and Koudinov A. R. (1996) Multiple inhibitory effects of Alzheimer's peptide Aβ-1-40 on lipid biosynthesis in cultured human HepG2 cells. *FEBS Lett.* **395,** 204–206.

Kounnas M. Z., Moir R. D., Rebeck G. W., Bush A. I., Argraves W. S., Tanzi R. E., Hyman B. T., and Strickland D. K. (1995) LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted β-amyloid precursor protein and mediates its degradation. *Cell* **82,** 331–340.

LaDu M. J., Falduto M. T., Manelli A. M., Reardon C. A., Getz G. S., and Frail D. E. (1994) Isoform-specific binding of apolipoprotein E to beta-amyloid. *J. Biol. Chem.* **269,** 23403–23406.

LaDu M. J., Pederson T. M., Frail D. E., Reardon C. A., Getz G. S., and Falduto M. T. (1995) Purification of apolipoprotein E attenuates isoform-specific binding to β-amyloid. *J. Biol. Chem.* **270,** 9039–9042.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lund-Katz S., Weisgraber K. H., Mahley R. W., and Phillips M. C. (1993) Conformation of apolipoprotein E in lipoproteins. *J. Biol. Chem.* **268,** 23008–23015.

Mahley R. W. (1988) Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* **240,** 622–630.

Mattson M. P., Wang H., and Michaelis E. K. (1991) Developmental expression, compartmentalization, and possible role in excitotoxicity of a putative NMDA receptor protein in cultured hippocampal neurons. *Brain Res.* **565,** 94–108.

Matz C. E. and Jonas A. (1982) Micellar complexes of human apolipoprotein A-I with phosphatidylcholines and cholesterol prepared from cholate-lipid dispersions. *J. Biol. Chem.* **257,** 4535–4540.

Motter R., Vigo-Pelfrey C., Kholodenko D., Barbour R., Johnson-Wood K. L., Galasko D., Chang L., Miller B., Clark C., Green R., Olson D., Southwick P., Wolfert R., Munroe B., Lieberburg I., Seubert P., and Schenk D. (1995) Reduction of the β-amyloid peptide (42), in the cerebrospinal fluid of patients with Alzheimer's disease. *Ann. Neurol.* **38,** 643–648.

Namba Y., Tomonaga M., Kawasaki H., Otomo E., and Ikeda K.

(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.

Narita M., Bu G., Holtzman D. M., and Schwartz A. L. (1997) The low-density lipoprotein receptor-related protein, a multifunctional apolipoprotein E receptor, modulates hippocampal neurite development. *J. Neurochem.* **68,** 587–595.

Paresce D. M., Ghosh R. N., and Maxfield F. R. (1996) Microglial cells internalize aggregates of the Alzheimer's disease amyloid beta-protein via a scavenger receptor. *Neuron* **17,** 553–565.

Pike C. J., Burdick D., Walencewicz A. J., Glabe C. G., and Cotman C. W. (1993) Neurodegeneration induced by β-amyloid peptides in vitro: the role of peptide assembly state. *J. Neurosci.* **13,** 1676–1687.

Pitas R. E., Boyles J. K., Lee S. H., Foss D., and Mahley R. W. (1987*a*) Astrocytes synthesize apolipoprotein E and metabolize apolipoprotein E-containing lipoproteins. *Biochim. Biophys. Acta* **917,** 148–161.

Pitas R. E., Boyles J. K., Lee S. H., Hui D., and Weisgraber K. H. (1987*b*) Lipoproteins and their receptors in the central nervous system. Characterization of the lipoproteins in cerebrospinal fluid and identification of apolipoprotein B,E(LDL) receptors in the brain. *J. Biol. Chem.* **262,** 14352–14360.

Poirier J. (1994) Apolipoprotein E in animal models of CNS injury and in Alzheimer's disease. *Trends Neurosci.* **17,** 525–530.

Poirier J., Hess M., May P. C., and Finch C. E. (1991) Astrocytic apolipoprotein E mRNA and GFAP mRNA in hippocampus after entorhinal cortex lesioning. *Mol. Brain Res.* **11,** 97–106.

Poirier J., Davignon J., Bouthillier D., Kogan S., Bertrand P., and Gauthier S. (1993) Apolipoprotein E polymorphism and Alzheimer's disease. *Lancet* **342,** 697–699.

Poirier J., Minnich A., and Davignon J. (1995) Apolipoprotein E, synaptic plasticity and Alzheimers disease. *Ann. Med.* **27,** 663–670.

Puttfarcken P. S., Manelli A. M., Falduto M. T., Getz G. S., and LaDu M. J. (1997) Effect of apolipoprotein E on neurite outgrowth and β-amyloid-induced toxicity in developing rat primary hippocampal cultures. *J. Neurochem.* **68,** 760–769.

Rebeck G. W., Reiter J. S., Strickland D. K., and Hyman B. T. (1993) Apolipoprotein E in sporadic Alzheimer's disease: allelic variation and receptor interactions. *Neuron* **11,** 575–580.

Saunders A. M., Strittmatter W. J., Schmechel D. E., St. George-Hyslop P. H., Pericak-Vance M. A., Joo S. H., Rosi B. L., Gusella J. F., Crapper-MacLachlan D. R., Alberts M. J., Hulette C., Crain B., Goldgaber D., and Roses A. D. (1993) Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease. *Neurology* **43,** 1467–1472.

Schmechel D. E., Saunders A. M., Strittmatter W. J., Crain B. J., Hulette C. M., Joo S. H., Pericak-Vance M. A., Goldgaber D., and Roses A. D. (1993) Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **90,** 9649–9653.

Selkoe D. J. (1994) Normal and abnormal biology of the β-amyloid precursor protein. *Annu. Rev. Neurosci.* **17,** 489–517.

Strittmatter W. J., Weisgraber K. H., Huang D. Y., Dong L. M., Salvesen G. S., Pericak-Vance M. A., Schmechel D. E., Saunders A. M., Goldgaber D., and Roses A. D. (1993) Binding of human apolipoprotein E to synthetic amyloid beta peptide: isoform-specific effects and implications for late-onset Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **90,** 8098–8102.

Swanson L. W., Simmons D. M., Hofmann S. L., Goldstein J. L., and Brown M. S. (1988) Localization of mRNA for low density lipoprotein receptor and a cholesterol synthetic enzyme in rabbit nervous system by in situ hybridization. *Proc. Natl. Acad. Sci. USA* **85,** 9821–9825.

Takadera T., Sakura N., Mohri T., and Hashimoto T. (1993) Toxic effect of a beta-amyloid peptide (beta 22-35) on the hippocampal neuron and its prevention. *Neurosci. Lett.* **161,** 41–44.

Takahashi S., Kawarabayasi Y., Nakai T., Sakai J., and Yamamoto T. (1992) Rabbit very low density lipoprotein receptor: a low density lipoprotein receptor-like protein with distinct ligand specificity. *Proc. Natl. Acad. Sci. USA* **89,** 9252–9256.

Weisgraber K. H. (1994) Apolipoprotein E: structure-function relationships. *Adv. Protein Chem.* **45,** 249–302.

Weisgraber K. H., Innerarity T. L., and Mahley R. W. (1982) Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site. *J. Biol. Chem.* **257,** 2518–2521.

Whitson J. S., Mims M. P., Strittmatter W. J., Yamaki T., Morrisett J. D., and Appel S. H. (1994) Attenuation of the neurotoxic effect of Aβ amyloid peptide by apolipoprotein E. *Biochem. Biophys. Res. Commun.* **199,** 163–170.

Wolf B. B., Lopes M. B., VandenBerg S. R., and Gonias S. L. (1992) Characterization and immunohistochemical localization of alpha 2-macroglobulin receptor (low-density lipoprotein receptor-related protein) in human brain. *Am. J. Pathol.* **141,** 37–42.

Yang D. S., Smith J. D., Zhou Z., Gandy S. E., and Martins R. N. (1997) Characterization of the binding of amyloid-β peptide to cell culture-derived native apolipoprotein E2, E3, and E4 isoforms and to isoforms from human plasma. *J. Neurochem.* **68,** 721–725.

Yankner B. A., Duffy L. K., and Kirschner D. A. (1990) Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. *Science* **250,** 279–282.

Zhou Z., Smith J. D., Greengard P., and Gandy S. E. (1996) Alzheimer amyloid-β peptide forms denaturant-resistant complex with type ε3 but not type ε4 isoform of native apolipoprotein E. *Mol. Med.* **2,** 175–180.