

Apolipoprotein E-containing High Density Lipoprotein Promotes Neurite Outgrowth and Is a Ligand for the Low Density Lipoprotein Receptor-related Protein*

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Presence of the $\epsilon 4$ allele of apolipoprotein E (apoE) is a risk factor for Alzheimer's disease (AD), although the mechanism(s) by which it confers this risk is unknown. ApoE may play a direct role in AD neuropathology by modulating neuronal structure. We previously showed that apoE3-containing β -very low density lipoprotein (β -VLDL) can stimulate neurite outgrowth to a significantly greater extent than apoE4-enriched β -VLDL in a central nervous system-derived neuronal cell line and that this effect is mediated by interaction with the low density lipoprotein receptor-related protein (LRP). To determine whether similar differences exist when apoE is associated with other lipoprotein particles, the effects of high density lipoprotein (HDL) derived from plasma and cerebrospinal fluid were defined. ApoE3-enriched HDL significantly enhanced neurite outgrowth as compared with apoE4-enriched HDL, and the majority of this stimulation was blocked in the presence of the receptor-associated protein or a neutralizing antibody to LRP. We also found that cholesterol esterification in the presence of apoE-containing plasma HDL was attenuated in fibroblasts lacking LRP. Therefore, apoE-containing HDL can serve as an LRP ligand, and apoE isoform-specific effects on neurite outgrowth are observed when HDL is the carrier particle.

Recent genetic epidemiologic studies have identified the $\epsilon 4$ allele of apolipoprotein E (apoE)¹ as a major risk factor for both sporadic and late-onset familial Alzheimer's Disease (AD) (1–5). The mechanism(s) by which the $\epsilon 4$ allele of apoE acts as a risk factor, however, remains unknown. It is possible that apoE plays a direct role in influencing the neurodegeneration and synaptic loss that occurs in the brains of patients with AD, since apoE is expressed in the normal and AD brain (6–10) and

its levels are up-regulated in response to brain injury (8–10). While little is known about the biological role of apoE in the normal, injured, or aging nervous system, recent *in vitro* data demonstrate that apoE-enriched β -very low density lipoproteins (β -VLDL) can modulate neurite outgrowth from neurons derived from both the peripheral nervous system (11–13) and central nervous system (CNS) (14). These effects have been shown to be isoform-specific, with apoE3-containing β -VLDL stimulating outgrowth to a greater extent than particles enriched with apoE4. Furthermore, the stimulatory effects of apoE-containing β -VLDL were shown to be mediated by the low density lipoprotein receptor-related protein (LRP) (14, 15), a lipoprotein receptor expressed at high levels in CNS neurons (16–18).

ApoE is a major apolipoprotein constituent in both brain and cerebrospinal fluid (CSF) (6, 7, 19, 20). In nervous tissue, lipoproteins and lipoprotein receptors are generated locally by different cell types. Whereas apoE is synthesized by nonneuronal glial cells (*i.e.* astrocytes) in the CNS (21, 22), LRP is expressed by neurons (16–18). In contrast to the situation in the circulatory system, apoE within the CSF is found within lipoprotein particles that are high density lipoprotein (HDL)-like in size (19, 20). ApoE in plasma is carried in VLDL, chylomicrons, and chylomicron remnants (23). The form in which apoE-containing lipoproteins are present in the brain have a direct bearing on the potential physiologic relevance of the effects of apoE3 *versus* apoE4 on neurons that have been reported *in vitro*, since most of these studies used apoE-containing β -VLDL particles rather than HDL-like particles that are most likely encountered by normal neurons *in situ*. Furthermore, apoE-containing HDL has not been reported to be a ligand for LRP, a prominent receptor for apoE in the brain. In the present study, we investigate these two issues. Using a previously characterized CNS-derived neuronal cell line (GT1-1 trk9) (14, 24, 25), we assessed whether apoE-enriched HDL particles obtained from plasma and CSF exhibit the same isoform-specific effects on neurite outgrowth that we originally observed using apoE-containing β -VLDL (14). We then investigated, using two different methods, whether apoE-enriched HDL is a ligand for LRP. First, we assessed whether the receptor associated protein (RAP) or neutralizing LRP antibodies were able to inhibit the neurite-promoting effects of apoE-enriched HDL; second, we tested the ability of these enriched particles to stimulate cholesterol esterification in fibroblasts expressing or lacking LRP (26). Our data demonstrate that 1) apoE-isoforms, when complexed to HDL, confer differential effects on CNS neurite outgrowth *in vitro*, 2) the neurite-promoting effects are predominantly mediated by LRP, and 3) LRP facilitates cholesterol esterification stimulated by apoE-containing HDL.

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¹ The abbreviations used are: apoE, apolipoprotein E; AD, Alzheimer's disease; β -VLDL, β -very low density lipoprotein; CNS, central nervous system; CSF, cerebrospinal fluid; HDL, high density lipoprotein; LDL, low density lipoprotein; LRP, low density lipoprotein receptor-related protein; NGF, nerve growth factor; RAP, receptor-associated protein; DMEM, Dulbecco's modified Eagle's medium.

MATERIALS AND METHODS

ApoE3 and ApoE4—We used apoE3 and apoE4 from three different sources: 1) recombinant human apoE3 and apoE4 purchased from PerImmune, Inc. (Rockville, MD), 2) human apoE3 and apoE4 purified from human plasma (gift from R. E. Pitas, B. Nathan, and R. W. Mahley), and 3) bacterially expressed recombinant human apoE3 and apoE4.

Expression and Purification of Bacterially Expressed Recombinant ApoE3 and ApoE4—ApoE3 and apoE4 cDNAs lacking the mammalian peptide signal sequence were amplified by polymerase chain reaction from the pCMV4-apoE3 and pCMV4-apoE4 expression vectors (gift of M. J. LaDu) and subcloned into the unique *Nde*I, *Bam*HI site of the pET-16b, His-Tag bacterial expression vector (Novagen, Inc., Madison, WI). The sequences of both cDNAs were verified by sequencing before utilization. Recombinant apoE3 and apoE4 were expressed in large quantities as N-terminal His-Tag fusion proteins in *Escherichia coli* strain BL21 (DE3). Expression of apoE proteins was induced with 1 mM isopropyl- β -D-thiogalactopyranoside for 45 min at 30 °C. Cell pellets were then harvested, and N-terminal His-Tag apoE3 and apoE4 were purified under denaturing conditions with nickel-nitrilotriacetic acid resin chromatography (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's instructions. After elution from the nickel-nitrilotriacetic acid resin with 8 M urea (in 0.1 M NaPO₄, 0.01 M Tris, pH 4.5), proteins were dialyzed extensively against 0.01 M NH₄HCO₃ at 4 °C and then concentrated with Amicon centricon concentrators (Amicon, Inc., Beverly, MA). We routinely obtained 1–2 mg of apoE/liter of bacterial culture. The biological effects of apoE produced in this fashion were indistinguishable from effects of the apoE purified from human plasma or from the preparations available commercially.

Recombinant 39-kDa RAP Protein and Anti-LRP Antibody—Purified recombinant RAP, anti-LRP immunoglobulin G (IgG), and nonimmune rabbit IgG were prepared as described (27, 28). Cells were preincubated in these reagents for 30 min at 37 °C before final addition of lipoproteins in all assays.

Neurite Outgrowth Assay—GT1-1 trk9 cells were grown as described previously (25). Cells (5000 cells/well) were plated in 96-well plates in serum-containing medium and allowed to attach overnight at 37 °C. The next day, cells were washed once with DMEM without serum and then incubated in serum-free N2 medium with or without: human nerve growth factor (NGF; 25 ng/ml; gift from D. Shelton, Genentech, Inc.), HDL (20 μ g cholesterol/ml), apoE3, or apoE4 (both 30 μ g/ml). Human plasma HDL ($d = 1.063$ – 1.21 g/ml) was isolated by sequential ultracentrifugation (29) and found to contain predominantly apoAI, as determined by Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis. Less than 1% of the protein content was apoE. Human CSF HDL ($d < 1.21$ g/ml) was isolated from 10 pooled CSF samples of heterogeneous apoE genotype (approximately 50 ml) as described (20) and concentrated with an Amicon centricon concentrator to a final cholesterol concentration of 250 μ g/ml. CSF HDL was found to contain apoAI and apoE, as described previously (19, 20). For the neurite outgrowth assay, HDL and the different apoE isoforms were incubated together for 30–60 min at room temperature before their addition to N2 medium. Cells were incubated with the indicated reagents for 24 h, after which they were fixed in 1% glutaraldehyde. A neurite was defined as a process with a length greater than or equal to one cell body in diameter. All cells encountered in a horizontal strip across the middle of each well using a $\times 20$ objective were evaluated (~ 100 – 200 cells/well, $n = 4$ wells per treatment condition). All measurements were done blind to the condition. The mean number of neurites per 100 cells for each condition was calculated, and results are presented as the percent differences between each treatment group and the base line within each experiment. The values for cells grown in NGF alone was set at 100% (base line). For experiments in which we quantified neurite length and mean cell body diameter, we visualized cells on a computer screen with a charge-coupled device camera and utilized the National Institutes of Health image program (version 1.55). All experiments were repeated three to five times. Data are presented as the means \pm S.E. Data were analyzed by analysis of variance and *t* tests with Bonferroni correction.

To confirm the validity of our neurite counting criteria, we compared our results to those obtained utilizing an image analysis system. We found that in no case were individual neurites we had counted using our original assessment criteria ever less than the calculated mean cell body diameter (beginning of a neurite was defined when the width of a process was ≤ 4 μ m). In addition, the mean number of neurites (expressed as percent HDL control) calculated according to our original criteria was virtually identical to that obtained having measured each of the neurites using the image analysis system (counting only those

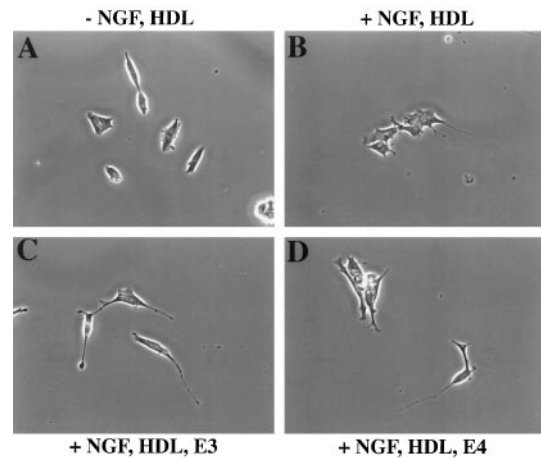


FIG. 1. Effects of apoE-containing plasma HDL on neurite outgrowth in GT1-1 trk9 cells. A, GT1-1 trk9 cells grown in serum-free N2 medium for 24 h in the absence of NGF do not extend neurites. B, addition of NGF (25 ng/ml) and HDL (20 μ g cholesterol/ml) causes an increase in neurite extension. C, apoE3-containing HDL and (D) apoE4-containing HDL both stimulate neurite outgrowth, but the effects of apoE3-containing HDL are greater than those of particles containing apoE4.

neurites ≥ 20 μ m, the calculated mean cell diameter). Thus, our counting criteria appear valid and reliable.

Cholesterol Esterification Assay—A wild-type fibroblast cell line (MEF-1) and those heterozygous (PEA-10) or homozygous (PEA-13) for the LRP-null mutation were a gift of T. Willnow and J. Herz (26). Cells were plated in 12-well tissue culture dishes in DMEM containing 10% (v/v) fetal calf serum, 2 mM glutamine, and penicillin/streptomycin (1000 units/ml) until they reached 90–95% confluence. They were then washed once with DMEM without serum and incubated in serum-free DMEM containing [³H]oleate complexed to bovine serum albumin (see below). Cells were evaluated after a 5-h incubation with selected lipoproteins, including low density lipoprotein (LDL) (10 μ g of protein/ml), HDL (20 μ g cholesterol/ml), apoE3, or apoE4 (both 30 μ g/ml). Where applicable, lipoproteins and the different apoE isoforms were incubated together for 30–60 min at room temperature before they were added to the medium.

Incorporation of [³H]oleate into cholesterol esters was determined as described previously (30). Each treatment condition was performed in duplicate or triplicate per assay, and a minimum of three assays were performed. Data are presented as the means \pm S.E.

RESULTS AND DISCUSSION

We sought to determine whether there are isoform-specific differences in the biological effects of apoE on CNS-derived neurons. We have shown previously that apoE3-enriched β -VLDL stimulates neurite outgrowth to a greater extent than apoE4-containing β -VLDL and that this stimulatory effect is mediated by LRP (14). However, given that apoE is normally found specifically within HDL-like (not VLDL) particles in CSF (19, 20), and perhaps in brain parenchyma itself, we hypothesized that HDL particles containing the different isoforms of apoE might also differentially stimulate neurite outgrowth, since this is likely to be a lipoprotein context similar to that in which neurons encounter apoE *in situ*.

Using our previously characterized neurite outgrowth assay (14), we observed that ~ 2 – 5 % of the neurons bore neurites in the absence of NGF. In the presence of NGF (25 ng/ml) for 24 h, this number increased to ~ 10 – 20 %. The percentage did not increase further on addition of native HDL at concentrations ranging from 10 to 80 μ g of cholesterol/ml. However, when cells were incubated in the presence of HDL that had been enriched with apoE, neurite outgrowth increased above the base line level observed for NGF alone (Figs. 1 and Fig. 2, A and B). In addition, the effect of apoE3 was significantly greater than that of apoE4 (Figs. 1 and 2, A and B).

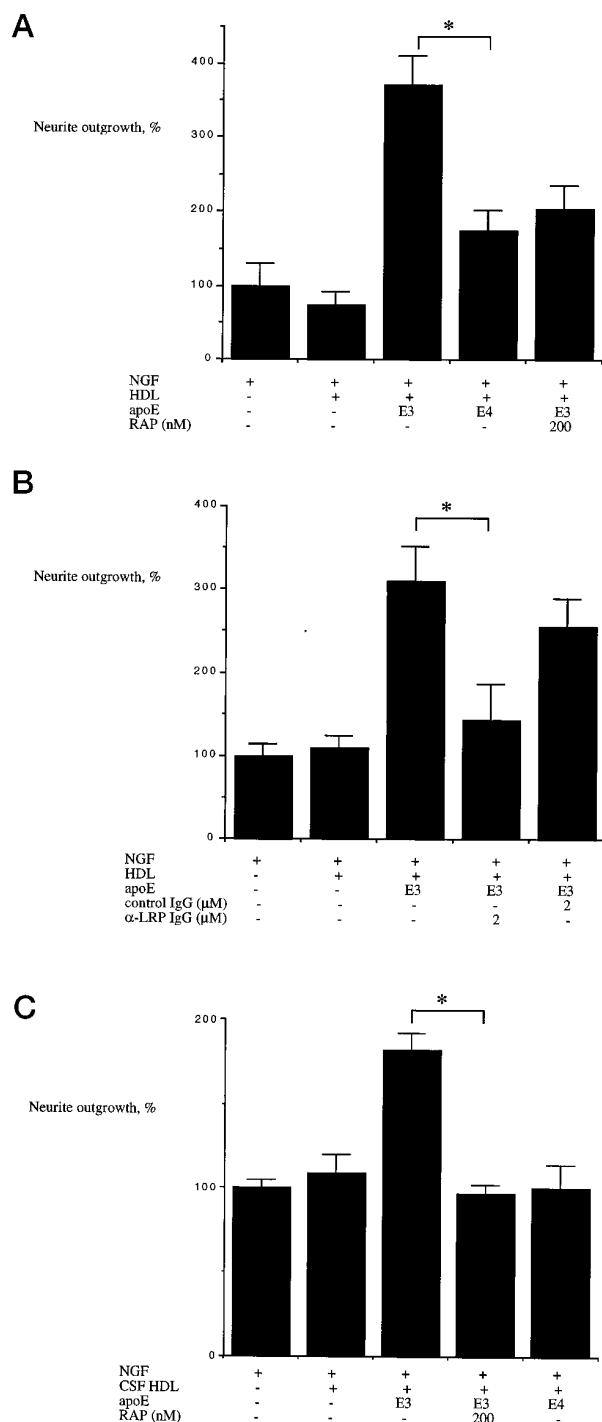


FIG. 2. Effects of NGF, plasma HDL, CSF HDL, apoE, RAP, and anti-LRP IgG on neurite outgrowth in GT1-1 *trk9* cells. The number of neurites per cell was assessed in individual wells (>100 cells/well) in the presence of the listed reagents. Value for cells grown in NGF (25 ng/ml) alone was set at 100% neurite outgrowth (base line). Percentage neurite outgrowth refers to the number of neurites per cell under experimental conditions as compared with base line. ApoE isoform concentration was 30 $\mu\text{g/ml}$, and HDL concentration was 20 μg cholesterol/ml for all experiments. $n = 4$ for all conditions. **A**, apoE3-containing plasma HDL enhanced neurite outgrowth to a significantly greater extent than did apoE4-containing HDL (*, $p < 0.05$). Most of the stimulatory effect of apoE3-containing HDL was blocked by RAP. **B**, the stimulation of neurite outgrowth by apoE3-containing plasma HDL was significantly inhibited by anti-LRP IgG (*, $p < 0.05$). **C**, apoE3-containing CSF HDL stimulated neurite outgrowth, whereas apoE4-containing particles did not. ApoE3-induced stimulation was blocked by RAP (*, $p < 0.001$). Bars represent S.E. In addition to counting number of neurites per cell, we also compared mean neurite length and the sum total of neurite lengths which were $\geq 20 \mu\text{m}$ (mean cell body diameter)

We also addressed the question of whether apoE-containing HDL exerts its neurite-promoting effects via LRP by performing the neurite outgrowth assay in the presence of anti-LRP IgG as well as the 39-kDa RAP, which inhibits all known ligand binding to LRP (31). The anti-LRP IgG does not recognize other closely related lipoprotein receptors (14, 27). Both RAP (200 nM) and anti-LRP (2 μM) inhibited ~ 60 – 70% of the neurite outgrowth stimulated by apoE3-enriched HDL (Fig. 2, A and B), indicating that the majority of the stimulatory effect of apoE-containing HDL in this cell culture system is mediated via interactions with LRP. Similar results were obtained in neurite outgrowth experiments utilizing HDL derived from human CSF (Fig. 2C); whereas CSF HDL alone had little effect on neurite outgrowth, significant stimulatory effects were observed when CSF HDL was enriched with apoE3 but not when it was enriched with apoE4. Furthermore, this apoE3-stimulated neurite outgrowth was blocked by RAP (Fig. 2C). Together these results suggest that apoE-containing HDL can serve as a ligand for LRP.

To further examine the possibility that apoE-containing HDL is a ligand for LRP, we investigated the ability of apoE-enriched plasma HDL to stimulate cholesterol esterification in wild-type (MEF-1) fibroblasts (LRP +/+) in the absence and presence of RAP. As shown in Fig. 3A, enrichment of HDL with apoE3 led to a significant increase in the level of cholesteryl [^3H]oleate formation in MEF-1 cells above that observed with HDL alone. These results were similar regardless of the specific apoE isoform used (apoE3 versus apoE4). Furthermore, this increase was blocked (74%) by RAP, suggesting that much of the stimulatory effect is mediated by interaction with LRP. To further address this possibility, we performed the cholesterol esterification assay using fibroblasts heterozygous (PEA-10; LRP +/-) or homozygous (PEA-13; LRP -/-) for the LRP-null mutation. Similar to what we observed with wild-type cells, HDL alone at concentrations of 10–40 μg of cholesterol/ml never significantly stimulated cholesterol esterification in either cell type. In contrast to native HDL, apoE3-enriched HDL stimulated cholesterol esterification in both PEA-10 and PEA-13 cells; however, the extent of stimulation was significantly greater in the LRP-expressing PEA-10 cells (Fig. 3B), providing further evidence that LRP can serve as a receptor for apoE-containing HDL. Of interest is the observation that apoE3- and apoE4-enriched HDL did not differ consistently in their ability to stimulate cholesterol esterification in PEA-10 or PEA-13 cells.

To test the possibility that the differential effects of apoE-enriched HDL in PEA-10 and PEA-13 cells could be mediated by differences in LDL-receptor function in the two cell lines, experiments were also performed in the presence of LDL. LDL ($d = 1.019$ – 1.063 g/ml) was isolated from human plasma and found to contain predominantly apoB100. PEA-10 and PEA-13 cells did not differ in their esterification response to LDL (Fig. 3B). This observation is consistent with a previous report demonstrating no difference between LRP-expressing and LRP-deficient fibroblasts in their ability to internalize and degrade ^{125}I -LDL (32). Thus, the differences in cholesterol esterification mediated by apoE-enriched HDL in these two cell types is unlikely to be due to differences in LDL-receptor function.

The present data demonstrate that apoE-containing HDL can serve as a ligand for LRP and that these particles can

in the presence of NGF/HDL or NGF/HDL/apoE3. The mean neurite lengths (\pm S.E.) under the two treatment conditions were: HDL = $45.5 \mu\text{m} \pm 12.9$ ($n = 29$) versus HDL/E3 = $61.9 \mu\text{m} \pm 30.1$ ($n = 54$), $p < 0.005$. The sum total of neurite lengths $\geq 20 \mu\text{m}$ were (mean percent HDL control \pm S.E.): HDL = $100\% \pm 17.0$ ($n = 29$) versus HDL/E3 = $253\% \pm 21.6$ ($n = 54$), $p < 0.001$.

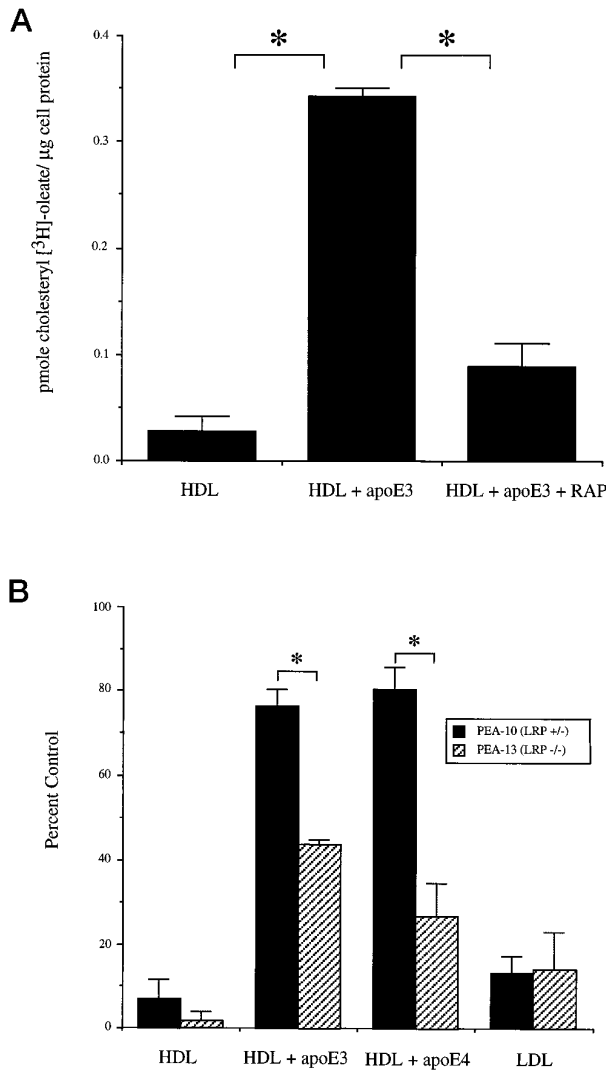


FIG. 3. Stimulation of [³H]oleate incorporation into cholesterol esters by apoE-enriched plasma HDL in mouse embryonic fibroblasts expressing different levels of LRP. *A*, plasma HDL enriched with apoE3-stimulated cholesterol ester synthesis in wild-type MEF-1 cells (LRP +/+) above the level observed with HDL alone (*, $p < 0.0001$). RAP blocked 74% of the apoE3-induced stimulation (*, $p < 0.0001$). *B*, apoE-enriched plasma HDL stimulated cholesterol esterification to a greater extent in PEA-10 (LRP ±; solid bars) than in PEA-13 cells (LRP -/-; hatched bars) (*, $p < 0.05$). These two cell lines did not differ in their esterification response to LDL. Data are expressed as percent of values obtained in wild-type MEF-1 cells upon stimulation with apoE3-containing HDL. Final concentrations for reagents were as follows: LDL, 10 μg of protein/ml; HDL, 20 μg of cholesterol/ml; apoE isoforms, 30 μg/ml; and RAP, 1 μM. Data in *A* are from one representative experiment, with $n = 3$ for all conditions. Data in *B* are from one representative experiment, with $n = 2$ for all conditions. Error bars correspond to S.E.

modulate neurite outgrowth in an isoform-specific manner. While previous observations of isoform-specific neurite-promoting effects of apoE-containing β-VLDL provided support for the concept of a potential deficiency in structural remodeling capability in the brains of patients with AD exhibiting the apoE4 genotype, the physiologic relevance of these findings was uncertain, since neurons of the CNS are unlikely to encounter such particles *in situ*. Our observations of differential neurite-promoting effects of apoE3- versus apoE4-containing HDL from plasma as well as CSF suggest that similar particles within the CNS could differentially influence neuronal structure and/or function in the normal and aging brain. We can thus hypothe-

size that the overrepresentation of the ε4 allele of apoE in patients with AD may confer a special vulnerability to otherwise normal age-related processes in the brain that require neuronal process remodeling, such as synaptic replacement and compensatory dendritic outgrowth, both of which are impaired in AD (33–36).

Our findings using apoE-containing CSF HDL on a CNS-derived neuronal cell line are consistent with those of Bellosta *et al.* (15) using bovine CSF on a neuroblastoma cell line and furthermore imply that direct effects of apoE-containing lipoproteins on CNS neurons *in vivo* may be mediated via LRP. The mechanisms underlying the differential effects of apoE3 versus apoE4 on neurite outgrowth, however, remain unclear. It is possible that there are differences in the ability of the apoE isoforms to stimulate cholesterol and/or lipid utilization in neurons or some other function(s) mediated by LRP and/or apoE that are unrelated to lipoprotein metabolism. Our data suggest that focusing attention on apoE-LRP interactions and their biological consequences, as well as on the mechanism(s) of isoform-specific effects, may lead to a better understanding of the role of these molecules in the normal brain and provide insight as to the relationship between the presence of the apoE4 isoform and the development of AD.

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