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Low density lipoprotein receptor-related protein mediates apolipoprotein E-dependent neurite outgrowth in a central nervous system-derived neuronal cell line

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ABSTRACT **The ε4 allele of apolipoprotein E (apoE) is a major risk factor for Alzheimer disease, suggesting that apoE may directly influence neurons in the aging brain. Recent data suggest that apoE-containing lipoproteins can influence neurite outgrowth in an isoform-specific fashion. The neuronal mediators of apoE effects have not been clarified. We show here that in a central nervous system-derived neuronal cell line, apoE3 but not apoE4 increases neurite extension. The effect of apoE3 was blocked at low nanomolar concentrations by purified 39-kDa protein that regulates ligand binding to the low density lipoprotein receptor-related protein (LRP). Anti-LRP antibody also completely abolished the neurite-promoting effect of apoE3. Understanding isoform-specific cell biological processes mediated by apoE–LRP interactions in central nervous system neurons may provide insight into Alzheimer disease pathogenesis.**

Recent genetic studies have identified the ε4 allele of apolipoprotein E (apoE) as a major risk factor in both sporadic and late-onset familial Alzheimer disease (AD) (1–3). The frequency of the ε4 allele is increased 2- to 3-fold in AD, whereas the presence of the ε2 allele appears to correlate with a longer life span (4, 5), suggesting possible neuroprotective effects. ApoE may play a role in influencing the neuronal degeneration and synaptic loss that occurs in the AD brain because it is expressed in both the normal and AD brain (6–8), as well as being markedly up-regulated in the injured brain (9). There is already direct evidence that apoE-containing lipoproteins can influence both lipoprotein uptake as well as neurite outgrowth *in vitro* (10, 11). Furthermore, recent data suggest that apoE-containing lipoproteins can influence neurite outgrowth in peripheral nervous system neurons in an isoform-specific fashion (12). ApoE may play a similar role in central nervous system (CNS) processes such as synaptic maintenance and the proliferation of new dendrites, normal processes in the aging brain (13). This may be particularly relevant to AD, in which there is both synaptic loss and failed compensatory dendritic outgrowth (13, 14).

In the CNS, apoE is expressed by astrocytes (6, 15) and is a major apolipoprotein in brain and cerebrospinal fluid (16, 17). The receptor(s) that mediates the effects of apoE on CNS neurons has not been defined. Two known cell-surface receptors can bind and initiate the endocytosis of apoE-containing lipoproteins, the low density lipoprotein (LDL) receptor (18) and the LDL receptor-related protein (LRP). LRP is a multifunctional cell-surface receptor that binds and "endocytoses" several distinct ligands, including apoE-enriched lipoproteins (19–23). LRP appears to be a good candidate for mediating the effects of apoE on CNS neurons because both the LRP and a 39-kDa protein, which copurifies with LRP and regulates its ligand binding (24), are expressed in neurons throughout the CNS (8, 25–27).

Although little is yet known about the functional role of apoE in the normal, injured, or aging CNS, data suggest that apoE can interact with neurons derived from the peripheral nervous system. *In vitro* experiments with PC-12 cells and dorsal root ganglion neurons have shown that apoE can mediate lipoprotein uptake through receptor-mediated endocytosis (10, 11). In addition, apoE together with β-migrating very low density lipoprotein (β-VLDL) can alter neurite morphology in rabbit dorsal root ganglion neurons (11). More recent work in this system has shown that human apoE3 increases neurite outgrowth, whereas apoE4 decreases neurite outgrowth (12). These effects were prevented by the addition of an antibody to apoE or by reductive methylation of the apoE (12). Taken together, these results suggest that the effects of apoE isoforms on neurite outgrowth in peripheral nervous system neurons may be mediated by lipoprotein receptors. To understand the molecular basis for these findings and to determine whether they are relevant to neurons derived from the brain, we have investigated the effects of apoE isoforms on an immortalized CNS-derived neuronal cell line, GT1-1 trk9 (28, 29).

MATERIALS AND METHODS

Cell Culture. GT1-1 trk9 cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum, 5% horse serum, penicillin (100 units/ml)/streptomycin (100 μg/ml), and Geneticin (300 μg/ml) as described (29).

Neurite Outgrowth Assay. GT1-1 trk9 cells (20,000 cells per well) were plated in 24-well tissue culture dishes in serum-containing medium and allowed to attach overnight at 37°C. The next day, cells were washed twice with phosphate-buffered saline (PBS) and incubated in N2 medium with or without the following additives: nerve growth factor (NGF), β-VLDL, apoE2, apoE3, or apoE4. NGF, β-VLDL, and human apoE were prepared and added to N2 medium exactly as described (12, 29). After 24 hr, cells were fixed in 2% glutaraldehyde and stored in water. To measure neurite outgrowth, cells were visualized with a ×20 objective with a phase-contrast microscope. A neurite was identified as a process of length greater than one cell body in diameter with a definable growth cone. In each well, 175–200 cells were assessed. Under all conditions

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Abbreviations: apoE, apolipoprotein E; AD, Alzheimer disease; LRP, low density lipoprotein receptor-related protein; NGF, nerve growth factor; diI, 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; LDL, low density lipoproteins; β-VLDL, β-migrating very low density lipoproteins.

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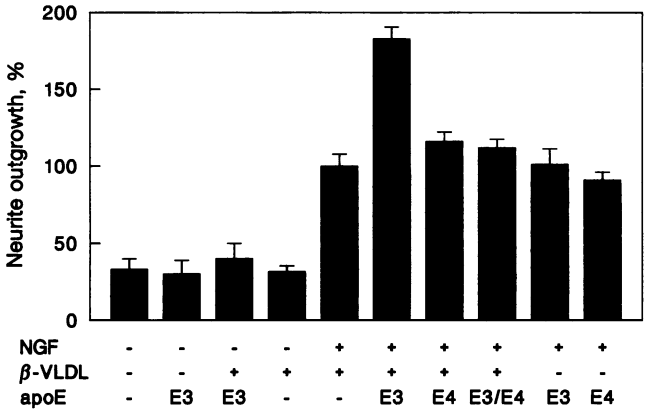

FIG. 1. Effect of NGF, β-VLDL, and apoE on neurite outgrowth in GT1-1 trk9 cells. In the presence of the listed reagents, the percentage of cells with neurites was assessed in individual wells. Value for cells grown in NGF (20 ng/ml) and β-VLDL (60 μg/ml) was set at 100% neurite outgrowth (baseline). Percentage neurite outgrowth refers to the percentage of cells with neurites seen under experimental conditions as compared to baseline. ApoE isoform concentrations were 30 μg/ml for all experiments shown. When we use criteria for neurites of processes greater in length than two cell body diameters, approximately half as many neurites are scored under all conditions. Results using these criteria differ slightly from those of Fig. 1 and are as follows (% neurite outgrowth): NGF/β-VLDL = 100 ± 14.8, n = 4; NGF/β-VLDL/apoE4 = 112 ± 10.3, n = 4; NGF/β-VLDL, apoE3 = 310 ± 21.7, n = 4; $P < 0.0001$ comparing condition with apoE3 vs. others.

tested here in the absence of NGF, ≈5% of cells bore neurites. In the presence of NGF (20 ng/ml) for 24 hr with or without β-VLDL, ≈15–20% of cells bore neurites. At this time point if a cell had neurites, there were usually one to three neurites per cell. Data were calculated as the percentage difference between each treatment group and the baseline for each experiment. Within experiments, the percentage differences for each condition were then averaged. The value for cells grown in NGF (20 ng/ml) and β-VLDL (60 μg of cholesterol/ml) was set at 100% (baseline). All experiments were repeated two to five times, and in individual experiments the data were repeated three or four times for each condition. Data are presented as means ± SEMs. The total number of cells was also assessed under all conditions tested, and there were no statistical differences. Neurite branching is minimal in GT1-1 trk9 cells and was not assessed. Data were analyzed by ANOVA and t tests with Bonferroni correction.

Recombinant 39-kDa Protein and Anti-LRP Antibody. Purified recombinant 39-kDa protein, anti-LRP IgG, and nonimmune rabbit IgG were prepared as described (30). Anti-LRP IgG and nonimmune rabbit IgG were dialyzed against Dulbecco's modified Eagle's medium before use in the neurite-outgrowth assay. When used in the neurite-outgrowth assay, reagents were added to N2 medium together with other additives at the beginning of each assay.

Chemical Crosslinking. Metabolic labeling, ligand binding, chemical crosslinking with DTSSP [3,3′-dithiobis(sulfosuccinimidylpropionate)], and immunoprecipitation were done as described (30, 31).

Indirect Immunofluorescent and Confocal Microscopic Localization of LRP and the 39-kDa Protein. GT1-1 trk9 cells were grown on glass coverslips for 24 hr in N2 medium with or without NGF (20 ng/ml). The following procedures were then done at room temperature. Cells were fixed with 4% (wt/vol) paraformaldehyde in phosphate-buffered saline/1 mM $CaCl_2$/0.5 mM $MgCl_2$ (PBS/$CaCl_2$/$MgCl_2$) for 20 min. After two washes with PBS/$CaCl_2$/$MgCl_2$, cells were permeabilized with

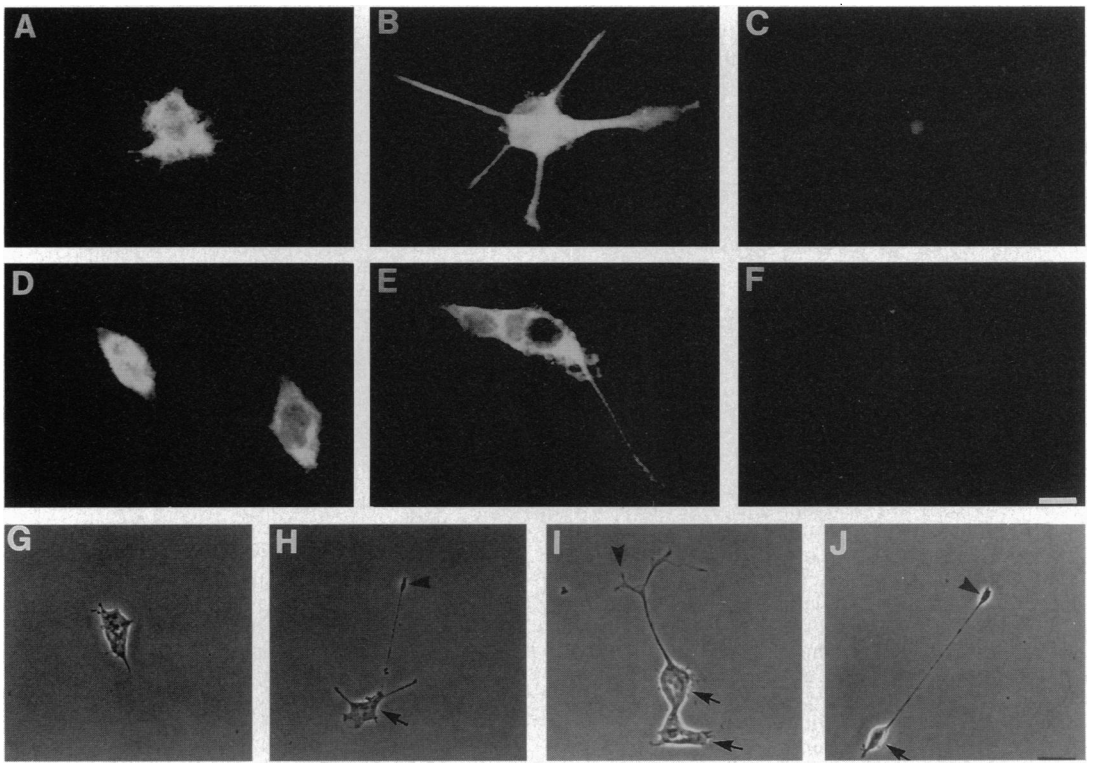

FIG. 2. GT1-1 trk9 cells express both LRP and the 39-kDa protein and develop neurites in response to NGF. By indirect immunofluorescence and confocal scanning microscopy, anti-LRP (*A* and *B*) and anti-39-kDa antibodies (*D* and *E*) stain cells grown without (*A* and *D*) or with (*B* and *D*) NGF. In *C* and *F*, the anti-LRP and anti-39-kDa protein antibodies were omitted from the staining procedure. Secondary antibodies labeled with rhodamine (LRP) or fluorescein isothiocyanate (39-kDa protein) were used to detect primary antibodies. For *A–F*, cells were grown on glass coverslips. (Bar = 10 μm.) By phase-contrast microscopy, well-developed neurites with growth cones (arrowheads) extend from some cell bodies (arrows) when cells are grown for 24 hr with (*H–J*) versus without (*G*) NGF. For *G–J*, cells were grown on tissue culture plastic. (Bar = 20 μm.)

1% (vol/vol) Triton X-100 in PBS/$CaCl_2$/$MgCl_2$ for 10 min before being blocked with 1% (wt/vol) bovine serum albumin/PBS/$CaCl_2$/$MgCl_2$/0.01% Tween 80 for 15 min. The primary antibodies (rabbit anti-LRP or rabbit anti-39-kDa protein) were prepared as described (30) and added at a 1:1000 dilution. After incubation at 37°C for 30 min, cells were washed with PBS/$CaCl_2$/$MgCl_2$ and then incubated with fluorescein isothiocyanate-coupled goat anti-rabbit IgG for 30 min (for 39-kDa protein) or with rhodamine-coupled goat anti-rabbit IgG (for LRP) for 30 min. The coverslips were then washed and mounted. Control labeling included each secondary antibody only. Sections were laser scanned using a Molecular Dynamics confocal microscope as described (30).

Epifluorescent Imaging and Confocal Microscopic Localization of 1,1-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (diI)-Labeled β-VLDL and LDL. GT1-1 trk9 cells were grown on glass coverslips for 24 hr in serum-containing medium in the presence of NGF (20 ng/ml). Cells were then washed twice with PBS/$CaCl_2$/$MgCl_2$ and incubated for 2.5 hr at 37°C in N2 medium in the presence of (*i*) diI-labeled β-VLDL (protein at 2 μg/ml), (*ii*) apoE3-(5 μg/ml) or apoE4-(5 μg/ml) enriched diI-labeled β-VLDL, or (*iii*) diI-labeled LDL (protein at 2 μg/ml). When anti-LRP IgG was used in the assay, it was added to the cells 10 min before as well as during incubation with diI-labeled lipoproteins. diI-labeling of lipoproteins was done exactly as described (10, 11).

RESULTS AND DISCUSSION

GT1 cells are immortalized hypothalamic neurons (28). These cells possess several neuronal-specific properties, including expression of neuron-specific structural proteins, proteins present in synaptic vesicles, and gonadotrophin-releasing hormone (28). These cells also have electrical properties of neurons; they have spontaneous action potentials and express a functional γ-aminobutyric acid type A receptor chloride-channel complex (32). GT1-1 trk9 cells are a subclone of GT1 cells that are stably transfected with the NGF receptor trkA (29). They demonstrate a dose-dependent increase in neurite outgrowth to NGF similar to that seen in PC-12 cells and in developing dorsal root ganglion neurons (29). We have found that these cells do not appear to differ in any way from their parent cell line in neuronal properties. They express neuronal-specific proteins as originally described (28, 32), including functional conductance channels (D.M.H. and A. Charles, unpublished work).

To determine whether apoE affects neurite outgrowth in GT1-1 trk9 cells, cultures were incubated in serum-free medium (N2 medium), in N2 medium/β-VLDL, or in N2 medi-

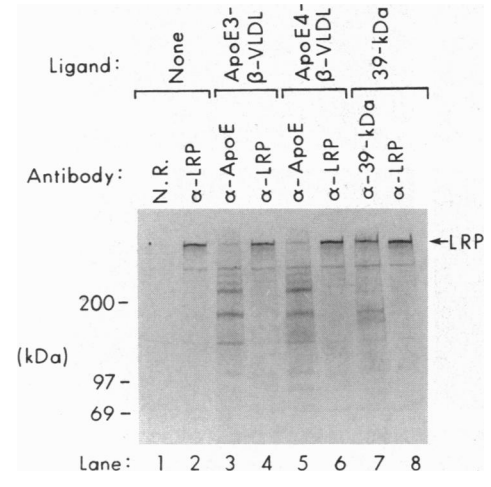

FIG. 3. Chemical cross-linking of apoE-enriched β-VLDL to cell-surface proteins on [^{35}S]cysteine-labeled GT1-1 trk9 cells. Cells grown in 10-cm dishes at ≈80% confluence were metabolically labeled with [^{35}S]cysteine (200 μCi/ml; 1 Ci = 37 GBq). After incubation for 4 hr at 37°C, cell monolayers were washed with prechilled binding buffer and incubated at 4°C in the same buffer with or without apoE3 (30 μg/ml)-enriched β-VLDL, apoE4 (30 μg/ml)-enriched β-VLDL, or 39-kDa protein (10 nM). After 1.5 hr of incubation, cells were crosslinked with 0.5 mM DTSSP. Cell lysates with or without ligand binding were immunoprecipitated with normal rabbit serum (N.R.) (lane 1), anti-LRP (lanes 2, 4, 6, 8), anti-apoE (lanes 3, 5), or anti-39-kDa protein (lane 7) and were analyzed by SDS/7% PAGE under reducing conditions. Position of the 515-kDa subunit of LRP is marked with an arrow.

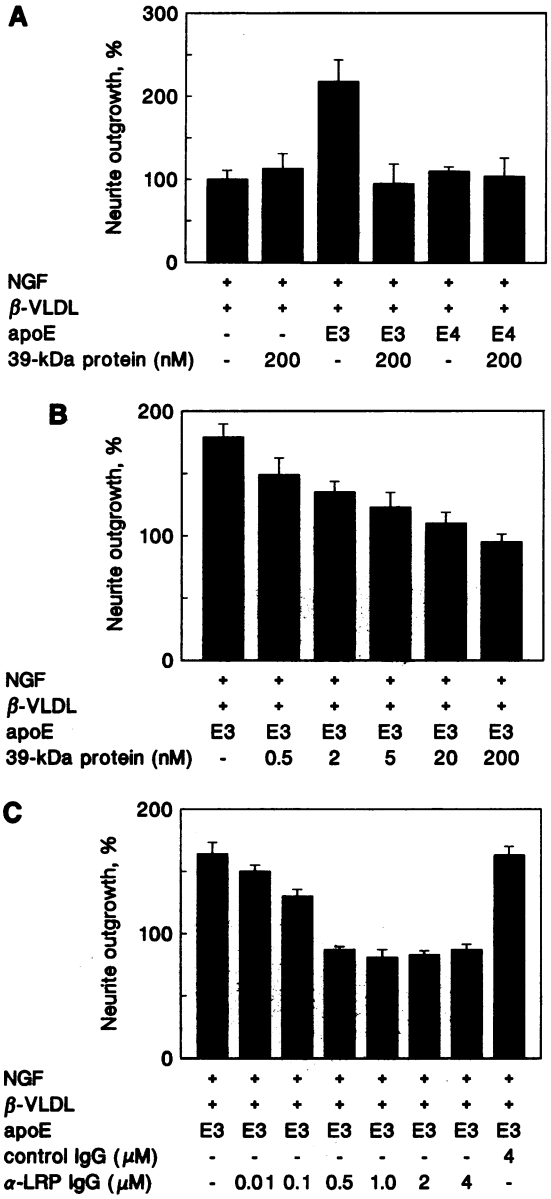

FIG. 4. The 39-kDa protein and an anti-LRP IgG both inhibit the apoE3-dependent increase in neurite outgrowth. At 200 nM, the 39-kDa protein completely inhibits apoE3-stimulated neurite outgrowth ($P < 0.001$ vs. NGF, β-VLDL, and apoE3) but had no effect on NGF-induced neurite outgrowth alone (*A*). The neurite-blocking effect of the 39-kDa protein was dose-dependent and reached statistical significance at 2 nM ($P = 0.02$) (*B*). The anti-LRP IgG also inhibited apoE3-induced neurite outgrowth in a dose-dependent way, and at 0.1 μM, the inhibitory effect was statistically significant ($P = 0.01$). At higher concentrations of anti-LRP IgG, the inhibitory effect was always significant ($P < 0.001$). (*C*) Concentrations of the control and anti-LRP antibody are given in micromolarity of IgG.

um/human apoE3 or apoE4 alone or N2 medium/human apoE3 or apoE4/β-VLDL. Twenty-four hours after treatment, cells were scored for neurites. Neurite outgrowth was present but minimal under all conditions without NGF (Fig. 1). As reported (29), NGF addition caused a dose-dependent increase in neurite outgrowth. In addition to NGF effects, apoE3/β-VLDL significantly enhanced neurite outgrowth ($P < 0.001$) (Fig. 1). If criteria for assessing neurite number were modified to include only processes greater in length than two cell body diameters, an even greater effect of apoE3-enriched β-VLDL was noted (Fig. 1, legend). Maximum effects were seen with β-VLDL (60 μg of cholesterol per ml) and apoE3 (30 μg/ml). Optimal enhancement of neurite outgrowth by apoE3 occurred at NGF concentrations of 20 ng/ml. With or without β-VLDL, apoE4 had no significant effect on neurite outgrowth (Fig. 1). In limited experiments, the effects of apoE2 were indistinguishable from those of apoE3 (data not shown), and the effects of both required β-VLDL (Fig. 1). Although there was no inhibitory effect of apoE4 on NGF-induced neurite outgrowth, simultaneous incubation of apoE3 and apoE4 with β-VLDL and NGF did not increase neurite outgrowth above that seen with NGF and β-VLDL alone (Fig. 1). Thus, as in previous work (12), apoE4 appeared to inhibit the neurite-promoting effects of apoE3. These results show an isoform-specific growth-regulatory function for apoE on CNS neurons and suggest the requirement of lipoprotein receptors.

Because CNS neurons *in vivo* appear to express LRP (8, 25–27) and the 39-kDa protein (27), we asked whether GT1-1 trk9 cells expressed these molecules. By indirect immunofluorescence, both proteins were localized to cell bodies and neurites (Fig. 2). To determine whether apoE directly associates with LRP on the surface of GT1-1 trk9 cells, we performed chemical cross-linking experiments. After metabolic labeling with [^{35}S]cysteine for 4 hr at 37°C, cells were incubated in N2 medium/NGF (20 ng/ml)/apoE3 or apoE4 (30 μg/ml)/β-VLDL (60 μg/ml) or in N2 medium/39-kDa protein (10 nM) for 1.5 hr at 4°C, cell-surface proteins were covalently crosslinked and thereafter immunoprecipitated from cell lysates with antibodies to LRP, to apoE, or to the 39-kDa protein as described (30). Antibodies to LRP (30) specifically immunoprecipitated the 515-kDa ligand-binding subunit of ^{35}S-labeled LRP (Fig. 3). Antibodies to apoE (12) and to the 39-kDa protein also immunoprecipitated LRP (Fig. 3). In addition to LRP, antibodies to apoE also immunoprecipitated several other proteins with lower molecular masses. One protein of ≈160 kDa may represent the LDL receptor.

To determine whether LRP plays a role in apoE3-induced neurite extension, we first asked whether the 39-kDa receptor-associated protein would inhibit this process. The 39-kDa protein copurifies with and specifically inhibits at low nanomolar concentrations binding and cellular uptake of all known ligands to LRP (33–35). In our neurite-outgrowth assay, the addition of purified recombinant 39-kDa protein completely inhibited the effect of apoE3 in a dose-dependent manner with an EC_{50} of ≈0.5 nM (Fig. 4 *A* and *B*). To test directly whether LRP was necessary for apoE-stimulated neurite extension, neurite-outgrowth assays were done in the presence of anti-LRP antibody (27, 30, 34). This antibody is specific for LRP, does not recognize the LDL receptor, and has previously been shown to completely block ligand binding to LRP on hepatoma cells (27, 30, 34). The antibody completely abolished the neurite-promoting effects of apoE3 (Fig. 4*C*). At higher concentrations, the antibody appeared to slightly decrease the effects of NGF-induced neurite outgrowth, but this result was not statistically significant. The antibody also blocked the neurite-promoting effects of apoE2 (data not shown). Nonimmune rabbit IgG had no effect on apoE3-stimulated neurite extension (Fig. 4*C*).

To further examine the potential mechanism by which anti-LRP antibody blocked the effects of apoE, we incubated GT1-1 trk9 cells with fluorescent-labeled β-VLDL in the presence or absence of apoE. With diI-labeled β-VLDL alone, a small amount of punctate fluorescence was seen in cells after 2.5 hr of incubation (Fig. 5). This pattern reflects receptor-mediated internalization of diI-labeled lipoproteins and the subsequent accumulation of unmetabolized diI in lysosomes (10). Qualitatively, the addition of apoE3- or apoE4-enriched diI-labeled β-VLDL resulted in a marked increase in the fluorescence pattern within cells (Fig. 5). This qualitative enhancement of intracellular diI-labeled β-VLDL accumulation was blocked by the addition of 1 μM anti-LRP IgG. Anti-LRP IgG (at concentrations as high as 5 μM IgG) did not

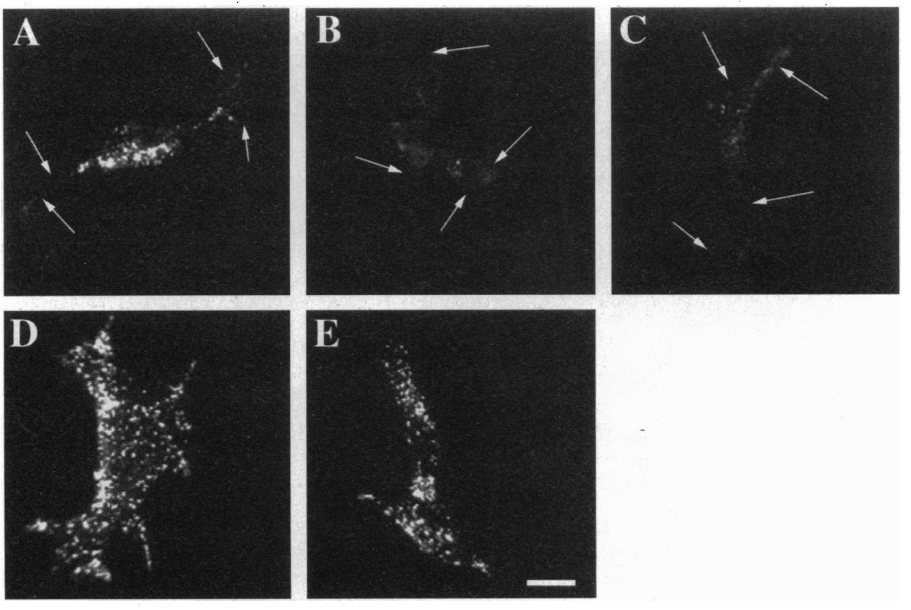

FIG. 5. Anti-LRP IgG qualitatively inhibits the apoE-stimulated increase in diI-labeled β-VLDL accumulation by GT1-1 trk9 cells but has no clear effect on diI-labeled LDL accumulation. In *A–E*, GT1-1 trk9 cells were imaged under epifluorescent illumination by confocal scanning microscopy after incubation for 2.5 hr in the presence of apoE3 enriched diI-labeled β-VLDL (*A*), diI-labeled β-VLDL alone (*B*), apoE3 enriched diI-labeled β-VLDL with 1 μM anti-LRP IgG (*C*), diI-labeled LDL (*D*), or diI-labeled LDL with 5 μM anti-LRP IgG (*E*). White arrows show cell borders. (Bar = 30 μm.)

appear to block the LDL-receptor-mediated intracellular accumulation of dil-labeled LDL by these cells (Fig. 5).

These results demonstrate that LRP mediates the neurite-promoting effects of apoE3-containing β-VLDL in a CNS-derived neuronal cell line. They also suggest that in these cells, LRP is in some way required for the isoform-specific growth-regulatory effects of apoE. In the cerebrospinal fluid, apoE is present in lipoprotein particles that are similar in density to high density lipoproteins in plasma (16). Whether apoE-containing lipoproteins within CNS tissue would exert similar effects on CNS neurons *in vivo* remains to be clarified. Future studies on primary CNS neurons both *in vitro* and *in vivo* will be important to evaluate further whether our findings can be generalized to other systems.

There are several possibilities as to why there are differences in the ability of apoE3 and apoE4 to augment neurite outgrowth. There may be apoE isoform-specific differences in (*i*) cell-surface access to LRP; (*ii*) binding to LRP; (*iii*) LRP-mediated endocytosis; or (*iv*) processes subsequent to endocytosis mediated by LRP. Because apoE effects on neurite outgrowth in CNS neurons may be directly relevant to synaptic and neuritic maintenance in the normal and AD brain, our data suggest that investigation into events surrounding or following apoE–LRP interactions within the CNS could yield important insights into the pathogenesis of AD.

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