8. Methods of surgery and extracellular recording were the same as those described previously (*6, 10*) and were conducted in accordance with institutional guidelines. In anesthetized bats, a metal post was attached to the skull, and a small hole was made in the skull overlying the inferior colliculus. After recovery from anesthesia, the bat was placed in a comfortable restraining device, and the post was secured to a stereotactic device. All physiological recordings were made while the bat was unanesthetized. The experiment was terminated if the bat showed any signs of discomfort. All parameters of the stimuli were controlled by computer and were generated by a digital-to-analog converter. The rise-fall times were 0.5 or 0.25 ms. The stimuli always started at the zero crossing point of the sine wave. Stimuli were presented at a rate of three per second. While searching for neurons, stimulus frequency and duration were varied; pure tones, FM sweeps, and noises were used. Twenty, 50, or 100 trials were presented at each stimulus duration tested, spike response times were digitized, and spike counts were recorded. To administer blockers of inhibitory transmitters, we glued a recording electrode to a five-barrel micropipette so that the tip of the recording electrode extended about 10 μm from the tip of the micropipette [for detailed description, see D. C. Havey and D. M. Caspary, *Electroencephalogr. Clin. Neurophysiol.* **48**, 249 (1980)]. Barrels were filled with 500 mM GABA (pH 3.5 to 4.0), 500 mM glycine (pH 3.5 to 4.0), 20 mM bicuculline methiodide (pH 3.0), or 20 mM strychnine HCl (pH 3.0 to 3.5). After collecting control data, we administered bicuculline or strychnine by iontophoresis (20 to 120 nA), the cell was tested again at the same stimulus durations, administration of the drug was terminated, the cell was tested for recovery at some sound durations, and the drug not administered in the first test was applied.
9. The best duration ranged from 1 to 30 ms for neurons that responded best to pure tones; most (20 out of 26) were tuned to durations of 5 ms or less. The best duration for neurons that responded best to FM sweeps ranged from 3 to 75 ms; most (13 out of 19) were tuned to durations between 3 and 8 ms. Although some best durations were longer than the longest echolocation sounds, they were still within the range of communication sounds. Isolation calls of infant *Eptesicus* can be over 100 ms in duration [E. Gould, *Commun. Behav. Biol. Part A Orig. Artic.* **5**, 263 (1971)].
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11. In three of the five neurons that were unaffected, we were able to test only the effects of blocking one inhibitory transmitter; thus, we cannot rule out the possibility that duration tuning would have been altered by application of the other drug. The duration tuning of the other two neurons was not affected by either drug. At this point, we do not know whether these two unaffected neurons reflect a technical problem in drug delivery or a real population of neurons that receive input from other neurons that are already tuned to duration.
12. Intracellular patch-clamp recordings were made from awake animals after a procedure similar to that described for in vivo recordings in the cortex of anesthetized animals [B. Jagadeesh, C. M. Gray, D. Ferster, *Science* **257**, 552 (1992)]. Animals were prepared as described (*8*). Electrode resistances ranged from 5 to 8 megohm. Electrodes were filled with a solution of 110 mM cesium gluconate, 11 mM EGTA, 10 mM $CsCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, 2 mM adenosine triphosphate, and 0.3 mM guanosine triphosphate. The pH was adjusted to 7.2; osmolarity was adjusted to 280 mosmol [E. Covey, B. R. Johnson, D. Ehrlich, J. H. Casseday, *Soc. Neurosci. Abstr.* **19**, 535 (1993)].
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17. R. B. Masterton has argued that brief sounds have a special biological importance for all mammals [*Trends Neurosci.* **15**, 280 (1992)]. Our results provide a neural definition of "brief."
18. We thank D. Caspary for instruction on iontophoretic drug application; J. A. Kauer for instruction on whole-cell patch-clamp recording; and B. Rosemond, C. Lucius, and S. Keros for technical assistance. B. R. Johnson, during the course of experiments on another topic (thesis, Duke University, Durham, NC, 1993), deserves credit for first finding a cell whose duration tuning was affected by antagonists of GABA or glycine. Supported by grants from NIH (DC-00287 and DC-00607) and NSF (IBN-9210299).

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Differential Effects of Apolipoproteins E3 and E4 on Neuronal Growth in Vitro

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Apolipoprotein E4 (apoE4), one of the three common isoforms of apoE, has been implicated in Alzheimer's disease. The effects of apoE on neuronal growth were determined in cultures of dorsal root ganglion neurons. In the presence of β-migrating very low density lipoproteins (β-VLDL), apoE3 increased neurite outgrowth, whereas apoE4 decreased outgrowth. The effects of apoE3 or apoE4 in the presence of β-VLDL were prevented by incubation with a monoclonal antibody to apoE or by reductive methylation of apoE, both of which block the ability of apoE to interact with lipoprotein receptors. The data suggest that receptor-mediated binding or internalization (or both) of apoE-enriched β-VLDL leads to isoform-specific differences in interactions with cellular proteins that affect neurite outgrowth.

Apolipoprotein E (apoE) is a 34-kD protein that mediates the binding of lipoproteins to the low density lipoprotein (LDL) receptor and to the LDL receptor–related protein (LRP) (*1, 2*). In the nervous system, apoE is synthesized and secreted primarily by astrocytes (*3, 4*) and macrophages (*5*). It is a major apolipoprotein in the brain (*6*) and in cerebrospinal fluid (*7*). Apart from its role in maintaining plasma cholesterol homeostasis, apoE is thought to participate in the mobilization and redistribution of lipids during normal development of the nervous system (*4*) and in the regeneration of peripheral nerves after injury (*8*). Three major isoforms of apoE (apoE2, apoE3, and apoE4) are products of three alleles (ε2, ε3, and ε4, respectively) at a single gene locus on chromosome 19 (*9*). The most common isoform is apoE3, which contains cysteine and arginine at positions 112 and 158, respectively. Both positions contain cysteine in apoE2 and arginine in apoE4.

The ε4 allele is specifically associated with both familial late-onset and sporadic Alzheimer's disease (AD), and the gene dose of ε4 is a major risk factor for the disease (*10*). Accumulated apoE is found in the senile plaques and neurofibrillary tangles seen in postmortem examinations of the brains of AD patients (*11*). Alzheimer's disease patients who are homozygous for the ε4 allele exhibit more highly developed senile plaques at autopsy than other AD patients (*12*). Thus, apoE4 may contribute to the pathogenesis of AD. Because neuronal pathology is a characteristic of AD, we examined the effect of human apoE on neurite outgrowth. Here we demonstrate that human apoE4 and apoE3 have different effects on the outgrowth of neurites from dorsal root ganglion (DRG) neurons in culture.

Rabbit DRG neurons were used as a model system to study the effects of lipids and apoE on neurite outgrowth. Previously, rabbit β-migrating very low density lipoproteins (β-VLDL), which are cholesterol-rich lipoproteins, have been shown to stimulate

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neurite branching (*13*). The addition of rabbit apoE (which is structurally related to human apoE3, as it contains cysteine in a location equivalent to position 112) to the cells with β-VLDL reduced neurite branching and increased neurite extension (*13*). The β-VLDL served as lipid-rich particles that transported apoE and allowed its interaction with the LDL receptor and with the LRP expressed by the neurons (*13, 14*). This study compared the effects of human apoE3 and apoE4 on neurite outgrowth.

Cultures of DRG neurons (*15*) were incubated for 18 to 20 hours in serum-free medium (N2 medium) alone, in N2 medium containing β-VLDL, or in N2 medium containing purified human plasma apoE3 or apoE4 alone or together with rabbit β-VLDL (*16*). Incubation with β-VLDL alone enhanced neurite growth, especially neurite branching (Fig. 1B), as compared with neurite growth observed in N2 medium alone (Fig. 1A). Human apoE3 with β-VLDL altered the growth pattern, resulting in an increase in neurite extension and a decrease in branching (Fig. 1C). In contrast to apoE3, human apoE4 with β-VLDL reduced both neurite extension and branching (Fig. 1D). Compared with β-VLDL alone, addition of as little as 0.6 μg/ml of apoE4 decreased neurite branching and extension ($P < 0.001$) (Fig. 2). On the other hand, apoE3 increased neurite extension and decreased branching (0.6 μg/ml, $P < 0.001$), as compared with β-VLDL alone. Thus, apoE3 may promote targeted neuronal growth in regeneration, whereas apoE4 may inhibit this process.

The inhibitory effect of apoE4 with β-VLDL on neurite growth was reversible. The removal of the apoE4 and β-VLDL from the cells in culture and the addition of apoE3 with β-VLDL or nerve growth factor promoted neurite outgrowth. In addition, the apoE4 effects on growth were dominant when the DRG neurons were simultaneously incubated with apoE3 (30 μg/ml), apoE4 (30 μg/ml), and β-VLDL (40 μg/ml) (Fig.

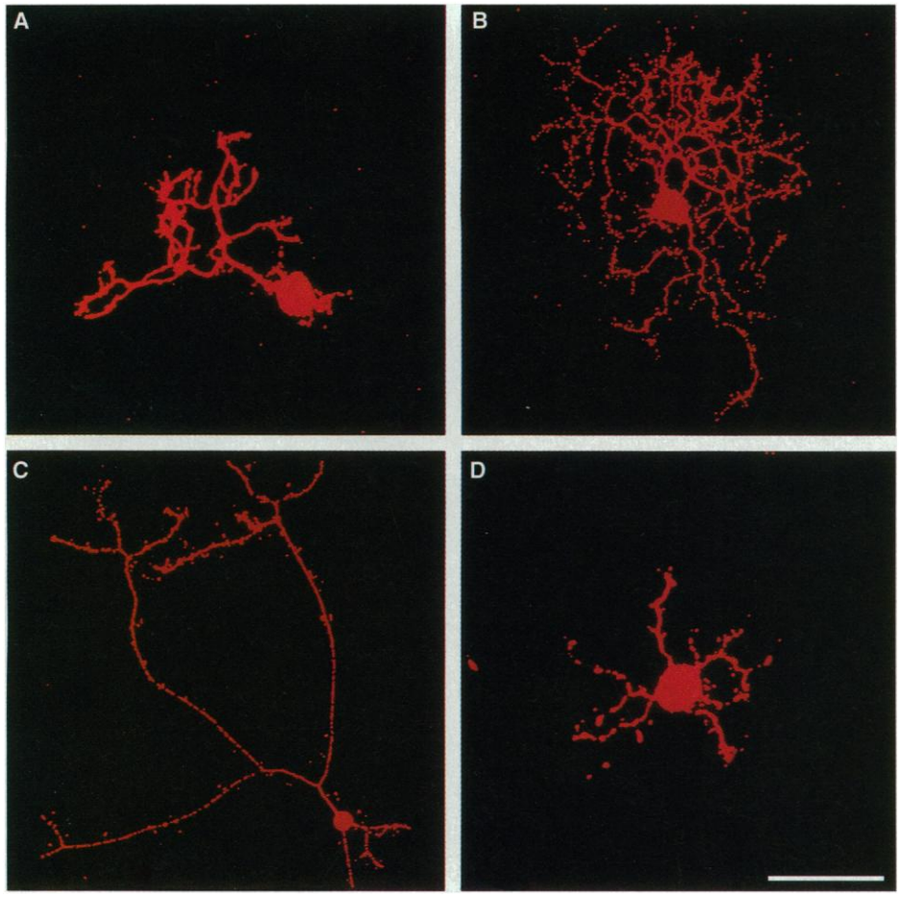

Fig. 1. Photomicrographs of representative DRG neurons grown (**A**) in N2 medium alone, (**B**) with β-VLDL (40 μg of cholesterol per milliliter), (**C**) with apoE3 (30 μg/ml) and β-VLDL (40 μg/ml), or (**D**) with apoE4 (30 μg/ml) and β-VLDL (40 μg/ml). Cells were nonspecifically stained with DiI (*13*). Scale bar, 100 μm.

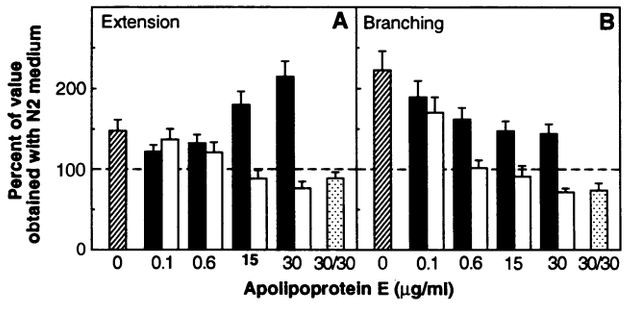

Fig. 2. Effect of β-VLDL (40 μg of cholesterol per mill- iliter) alone (diagonally striped bars), or together with either apoE3 (solid bars) or apoE4 (open bars) or both (dot-filled bars; 30 μg of each) on (**A**) neurite extension and (**B**) branching. For each variable, 30 to 40 treatment-responsive neurons were measured as described (*15*), and the data were calculated as the percent difference between each treatment group and the matched control (N2 medium alone) for each experiment. The percent differences for the different experiments were then averaged. The value for the N2 medium alone was set at 100% (dashed line). Data are presented as the mean ± SEM. Approximately 15 to 20% of the neurons did not respond to the treatments and were similar to control neurons. These cells may represent a neuronal subclass or injured neurons (*27*).

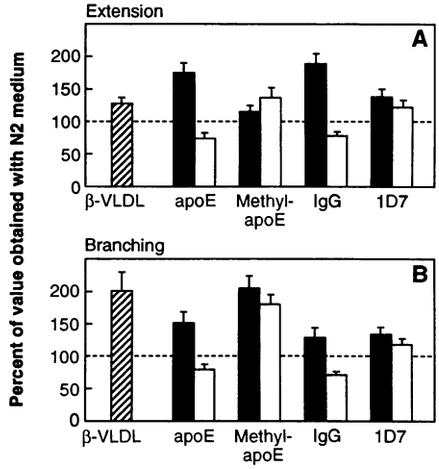

Fig. 3. The effect of apoE3 (30 μg/ml) (solid bars) or apoE4 (30 μg/ml) (open bars) in the presence of β-VLDL (40 μg of cholesterol per milliliter) on (**A**) neurite extension and (**B**) branching in the presence of the monoclonal antibody 1D7 (30 μg/ml), nonimmune mouse immunoglobulin G (IgG) (30 μg/ml), or apoE that had undergone reductive methylation (methyl-apoE, 30 μg/ml). The effect of β-VLDL alone is also shown (diagonally striped bars). For each variable, 20 to 30 neurons were measured and analyzed as in Fig. 2. The value for the N2 medium alone was set at 100% (dashed line).

2). These results demonstrate an isoform-specific, neuronal growth–regulatory function for apoE.

Apolipoprotein E–enriched β-VLDL bind to and are internalized by neurons in DRG cultures through LDL receptor– and LRP-mediated endocytosis (*13*). Apolipoprotein E is a ligand for these receptors only when it is present on lipoproteins (*1*, *2*). To determine if apoE binding to lipoprotein receptors is required for modulation of neurite outgrowth, we incubated the DRG neurons with apoE3 or apoE4 without β-VLDL. Neither apoE3 nor apoE4 by itself had an effect on neurite branching or extension (*17*). Monoclonal antibody 1D7, which inhibits apoE binding to lipoprotein receptors (*18*), and reductive methylation of apoE, which modifies lysine residues and also inhibits receptor interaction (*19*), were tested for their abilities to alter the effects of apoE3 and apoE4 on neurite growth. Both antibody 1D7 and reductive methylation of apoE abolished the differential effects of apoE3 and apoE4 with β-VLDL on neurite extension and branching (Fig. 3), whereas nonspecific immunoglobulin G did not (*20*). Thus, the effects of apoE are most likely mediated through receptor interaction with the cultured DRG cells.

Support for the idea that the different isoforms of apoE have a direct effect on neurite outgrowth comes from studies of a murine neuroblastoma cell line (Neuro-2a) that has been stably transfected with human apoE3 or apoE4 (*21*). When these transfected cells, which secrete nanogram quantities of human apoE, were incubated with β-VLDL, they displayed the same differential growth pattern as DRG neurons incubated with exogenous human apoE3 or apoE4 and β-VLDL.

The mechanism underlying the isoform-specific growth-regulatory effect of apoE may require the interaction of apoE with the LDL receptor, with LRP, or with other cell-surface molecules [such as heparan sulfate proteoglycans, which are known to bind apoE-enriched lipoprotein (*22*)]. Binding of specific isoforms of apoE to cell-surface receptors may signal intracellular events. On the other hand, internalized apoE3 or apoE4 may differentially affect intracellular processes that are responsible for the regulation of neurite extension and branching patterns. The presence of apoE has been demonstrated in a variety of intracellular locations, including endosomes, peroxisomes, and cytoplasm (*23*). Several isoform-specific interactions of apoE with cellular proteins, including proteins implicated in AD, have also been demonstrated (*24*–*26*). Apolipoprotein E4 interacts more readily than apoE3 with the amyloidogenic fragment of the amyloid precursor protein (*24*). In contrast, apoE4 does not bind to the microtubule-associated protein tau, a major component of neurofibrillary tangles, whereas apoE3 binds strongly (*26*). Binding of apoE3 to tau has been postulated to affect tau metabolism, possibly influencing tau phosphorylation and the formation of paired helical filaments present in neurofibrillary tangles (*26*). Because tau promotes microtubule assembly and stabilizes microtubules, differential isoform-specific interactions of apoE3 and apoE4 with tau may account for their differential effects on neurite outgrowth. The data suggest that isoform-specific effects of apoE with tau or other cellular proteins may play a role in the development of AD.

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15. Dorsal root ganglion neurons from fetal rabbits (at 25 days of gestation) were isolated (*13*) and purified (*27*) as described, except that 100-μm and 40-μm sieves were used instead of 150-μm and 10-μm sieves, respectively. Cultures of DRG neurons were 60 to 70% neurons. The purified neuronal suspension was diluted with N2 medium and plated (3000 cells in 200 μl) in the center of a 35-mm tissue culture plate coated with extracellular matrix (Accurate Chemical and Scientific, Westbury, NY). The cultures were maintained in a humidified incubator at 37°C and 5% CO_2. Two hours after plating, N2 medium (1 ml) and the test reagents were added to each dish. After 18 to 20 hours of incubation, the cells were nonspecifically stained with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI) and fixed with 4% paraformaldehyde. Neurons were imaged in fluorescence mode with a confocal laser scanning system (MRC-600, Bio-Rad, Hercules, CA), and the images were digitized with an Image-1/AT image analysis system (Universal Images, West Chester, PA). The neuronal images were coded before characterization, and the following variables were measured for each neuron: (i) neurite branching (the number of branch points), and (ii) neurite extension (the radial distance from the cell body to the end of each neurite). These studies were done with 10 different preparations of DRG neurons and with 5 independent preparations of apoE3 and apoE4. Similar results were obtained with all preparations.
16. Human apoE was purified from the plasma of apoE3 and apoE4 homozygotes, and biological activity was assessed by LDL-receptor binding assay after reconstitution with phospholipid (*28*). The β-VLDL were isolated as described (*29*). Human apoE3 or apoE4 was incubated alone or together with β-VLDL for 30 min at 23°C before being added to the cells in N2 medium. Under these conditions, endogenous rabbit apoE is essentially replaced by human apoE3 and apoE4. There is no indication that apoE3 or apoE4 can be differentially inactivated or denatured under the assay conditions. Both isoforms of human apoE are incorporated equally well into β-VLDL and are effective in stimulating the uptake of β-VLDL by the LRP (*29*).
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20. In cells incubated with methyl-apoE and β-VLDL, the effect of the added apoE was totally blocked, and neurite branching and extension were similar to those observed with β-VLDL alone (Fig. 3). With antibody 1D7, the differential effects of apoE3 and apoE4 were abolished; however, only neurite extension was similar to that shown by cells incubated with β-VLDL alone, whereas branching was reduced (Fig. 3). The reason for this differential response in the presence of methyl-apoE and antibody 1D7 is not known.
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