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Effects of Apolipoprotein E on Neurite Outgrowth from Adult Mice Cortical Neurons

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Effects of Apolipoprotein E on Neurite Outgrowth

from Adult Mice Cortical Neurons

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Yanwen Jiang

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Have not been supported by my parents and my sister, I would not have been able to complete this thesis. I would like to say sincerely thanks to them for their understanding and encouragement. I would like to thank Dr. Nathan, my advisor, who guided me through the last two years and introduced me to the wonderful world of research. I also would like to thank my thesis committee members, Dr. Kruse who helped me with my statistical analysis and Dr. Wong who greatly helped me out with immunocytochemistry and microscopy. I want to take this chance to thank my lovely labmates, Rafia Nisar, Sara Ludwig, John Yost, and Steve Slowik, whom I shared the happiness and sadness together. Finally, thanks to Dr. Brewer and Mr. John Torricelli at Southern Illinois University Medical School for their helpful suggestions.

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Abstract:

Apolipoprotein (apo) E4, one of the three common isoforms of apoE, has been implicated in Alzheimer's disease (AD). The mechanism whereby apoE4 leads to the pathogenesis of AD is unknown. In the present study, I examined the effects of apoE on neurite outgrowth from adult mice cortical neurons (AMC) in culture. I found that neurite outgrowth from AMC neurons derived from apoE deficient/apoE gene knockout (apoE KO) mice is significantly shorter than that from age-, sex-, and strain-matched wild-type control mice. Furthermore, I present evidence for the differential effects of two isoforms of human apoE, apoE3 and apoE4, on neurite outgrowth from apoE KO neurons. Human apoE3 enhances neurite outgrowth whereas human apoE4 inhibits outgrowth. The differential effects of apoE3 and apoE4 on neurite outgrowth appear to be mediated by the low-density lipoprotein receptor related protein (LRP). Blocking of the LRP pathway of lipoprotein uptake with lactoferrin and receptor associated protein (RAP) abolished the differential effects of apoE isoforms on neurite outgrowth. Further understanding of the isoform specific cell biological processes mediated by apoE-LRP interactions in AMC culture may provide insight into AD pathogenesis.

Introduction:

Apolipoprotein E (apoE) is a 299 amino acid protein (MW=34,200) encoded by a single gene located on chromosome 19 in human (Rall *et al.*, 1982; Das *et al.*, 1985; Paik *et al.*, 1985). The gene is highly polymorphic. In human, among all the variants, there are three major isoforms of apoE, apoE2, apoE3, and apoE4 that are produced by three alleles (e2, e3, and e4). The frequencies of these three alleles in the general population are 8%, 77%, and 15%, respectively (Mahley, 1988; Gooch and Stennett, 1996). The most common isoform, apoE3, has cysteine and arginine at positions 112 and 158. Both positions contain cysteine in apoE2, and arginine in apoE4 (Rall *et al.*, 1982; Weisgraber, 1994; Mahley *et al.*, 1995). Mice have one form of apoE which resembles human apoE4 by having arginine at both positions 112 and 158, but differs significantly in amino acid sequence at the carboxyl terminus (Weisgraber, 1994).

ApoE functions in the redistribution of lipids among various tissues by interacting with lipoprotein receptors on target cells, and thus apoE plays a critical role in lipid metabolism. In the liver, the uptake of chylomicron remnants via low density lipoprotein (LDL) receptor-related protein (LRP) has been known to be mediated by apoE (Kowal *et al.*, 1989, 1990). The clearance of very low-density lipoproteins (VLDL) and intermediate density lipoprotein (IDL) by the liver is also mediated by apoE through the LDL receptor. ApoE has also been suggested to play a role in the process of converting IDL to LDL by hepatic lipase. After LDL particles are produced, they are cleared by the liver via apoB100 through the LDL receptor (Bradley et al. 1984; Weisgraber, 1994). In addition, the excessive cholesterol and other lipids in some cells may form complexes

with high density lipoprotein (HDL) containing apoE that can transport the HDL to other cells or to the liver for clearance (Weisgraber, 1994; Mahley *et al.*, 1995).

In humans the brain is second only to the liver in apoE production (Elshourbagy *et al.*, 1985; Beffert *et al.*, 1998). In the central nervous system (CNS), apoE is synthesized and secreted primarily by astrocytes, and to a lesser extent, by macrophages (Elshourbagy *et al.*, 1985; Pitas *et al.*, 1987a). Within the cerebrospinal fluid, apoE is found associated with high density lipoprotein (HDL)-size lipoprotein particles and appear to play a major role in lipid transport in the CNS (Pitas *et al.*, 1987b; Mahley *et al.*, 1995; Beffert *et al.*, 1998). ApoE-containing lipoproteins bind to several lipoprotein receptors in the CNS, including the LDL receptor, the LDL receptor-related protein (LRP), the VLDL receptor, the glycoprotein (gp) 330, and apolipoprotein E receptor 2 (apoER2) (Herz *et al.*, 1988; Kowal *et al.*, 1990; Takahashi *et al.*, 1992; Willnow *et al.*, 1992; Beffert *et al.*, 1998). These receptors are members of a single family of proteins that share structural and functional similarities. The LDL receptor and LRP are expressed by neurons (Pitas *et al.*, 1987b). Previous studies have shown that apoE plays a pivotal role in maintaining central nervous system function and synaptic plasticity, especially after neuronal damage. ApoE levels dramatically increase after brain damage. For example, the expression of apoE increases following optic nerve injury in rats (Ignatius *et al.*, 1986). Furthermore, results from our laboratory have shown that apoE secretion increases 2-fold following olfactory nerve lesion in mice. It has also been shown that apoE mRNA expression in astrocytes in the hippocampus increases after entorhinal cortex lesion (Poirier *et al.*, 1991). ApoE appears to participate both in the scavenging of lipids generated after axon

degeneration and in lipid redistribution to sprouting neurites and to Schwann cells (Ignatius *et al.*, 1987; Mahley *et al.*, 1995).

Numerous recent studies have demonstrated that inheritance of the e4 allele of apoE increases the risk of Alzheimer's disease (AD) and that the inheritance of the e2 allele decreases the risk. Alzheimer's disease is the major form of dementia, and is characterized by senile plaques and neurofibrillary tangles in the brain and loss of cholinergic neurons in the basal forebrain (Gooch and Stennett, 1996). The senile plaques in the brains of AD patients consist of amyloid deposits surrounded by dystrophic axons. The amyloid is referred to as β-amyloid (Aβ), which is a product of inappropriate cleavage from the amyloid precursor protein (APP) (Haass and Selkoe, 1993; Gooch and Stennett, 1996; Vassar *et al.*, 1999). The second major type of brain lesion in AD patients, the neurofibrillary tangles, are located in cell bodies and apical dendrites. The tangles contain paired helical filaments composed of abnormally phosphorylated tau proteins. Tau is one of the microtubule-associated proteins (MAPs) that stabilizes microtubules against disassembly and provides a mechanism for them to interact with other cell components (Gooch and Stennett, 1996).

There are three forms of AD: early-onset familial AD, late-onset familial AD and sporadic AD. Early-onset familial AD, which accounts for only 5% of human patients, has been found to be related to mutations on chromosomes 1, 14, and 21 (Gooch and Stennett, 1996). The late-onset familial AD and sporadic AD, which accounts for approximately 95% of all late-onset AD cases, are associated with the inheritance of e4 (Poirier *et al.*, 1993; Beffert *et al.*, 1998). About 80% of familial and 64% of sporadic late-onset AD cases carry at least one copy of the e4 allele compared to 31% of controls

(Corder *et al.*, 1993; Beffert *et al.*, 1998). Furthermore, the e4 allele also has a strong impact on the age of onset of clinical symptoms in AD. Studies have shown that the average age at onset of AD patients without e4 allele is 84.3 years, compared to 75.5 years with individuals with a single e4 allele, and 68.4 years in individuals with two copies of e4. Most importantly, the survival time also decreases with an increasing gene dose of apoE4 (Corder *et al.*, 1993, Gooch and Stennett, 1996). These studies have been confirmed by many laboratories worldwide, and is now universally accepted that the apoE genotype is the major risk factor for AD. Unfortunately the mechanism whereby apoE4 leads to the pathogenesis of AD is unknown.

Previous findings from our laboratory have shown that apoE isoforms differentially alter neurite outgrowth from both CNS and PNS neurons. In the presence of a source of lipid, purified human apoE3 increased neurite extension, whereas human apoE4 decreased extension from cultured dorsal root ganglion neurons (DRG) (Nathan *et al.*, 1994). Similar results were observed in murine neuroblastoma cells (Neuro-2a) derived from the CNS (Bellosta *et al.*, 1995; Nathan *et al.*, 1995). These studies demonstrated that the effects of apoE isoforms on neurite outgrowth required the interaction of apoE with lipoprotein receptors. In DRG culture, the apoE isoform-specific effect on neurite outgrowth was abolished by addition of an antibody, 1D7, and also by reductive methylation of critical lysine residues in apoE (Nathan *et al.*, 1994), both of these approaches inhibit apoE binding to lipoprotein receptors. Furthermore, in both DRG culture and Neuro-2a culture, a lipid source was required to initiate the apoE effects. Addition of free apoE3 or apoE4 without a lipid source had no effect on neurite

outgrowth (Nathan *et al.*, 1994; Nathan *et al.*, 1995) suggesting that the effects of apoE on neurite outgrowth are lipoprotein receptor-mediated.

Among all the receptors known to bind apoE containing lipoproteins, two receptors: the LDL receptor, and the heparan sulfate proteoglycan (HSPG)-LRP have been very well studied (Herz *et al.*, 1988; Kowal *et al.*, 1990; Takahashi *et al.*, 1992). In studies on the role of apoE on chylomicron remnant clearance, evidence has shown that although the LDL receptor may participate in the uptake of chylomicron remnants, it is not essential for the rapid clearance of the remnants (Ji *et al.*, 1993). In contrast, previous studies have indicated that LRP is involved in the clearance of apoE-enriched remnants (Kowal *et al.*, 1990; Ji *et al.* 1993). Ji et al. (1993) demonstrated that the LRP and the cell-surface HSPG are jointly involved in the binding and internalization of apoE containing lipoproteins. Cultured CHO cells treated with heparinase demonstrated a remarkable decrease on the uptake of fluorescently labeled β-VLDL+apoE (Ji *et al.* 1993; Mahley *et al.*, 1994). Lactoferrin, a 76-kD glycoprotein which contains a cluster of four arginine residues resembling the receptor-binding domain of apoE (Metz-Boutigue *et al.*, 1984), bind to HSPG and LRP, and inhibit the plasma clearance of chylomicron remnants (Ji and Mahley, 1994). These results suggest that lipoproteins bind to HSPG prior to internalization by the LRP (Ji *et al.* 1993; Ji and Mahley, 1994). Previous studies have shown that receptor-associated protein (RAP, also called 39-kDa protein), which co-purifies with LRP also inhibits the binding of apoE containing lipoproteins to LRP (Williams *et al.*, 1992; Battey *et al.*, 1994).

How apoE isoforms differentially alter neurite outgrowth remains unclear, as is the relevance of these findings to adult neurons and Alzheimer's disease. The primary aim

of this study is to answer the second question by thoroughly examining the effects of apoE isoforms on neurite outgrowth from cortical neurons derived from the brains of adult mice. In this thesis, I demonstrate that (1) AMC neurons derived from apoE-deficient mice (apoE K.O.) have significantly shorter neurite outgrowth than neurons derived from normal mice; (2) human apoE3 enhances and human apoE4 inhibits neurite outgrowth in AMC neurons derived from apoE K.O. mice; and (3) the differential effects of apoE isoforms on neurite outgrowth in AMC neurons appears to require the LRP.

Methods and Materials

Materials:

AMC culture: Homozygous apoE KO mice (C57BL/6J-Apoe$^{<tmiUnc>}$, Cat. # 002052) and control mice (C57BL/6J, Cat. # 000664) were obtained from Jackson Laboratory (Bar Harbor, MA). Cell culture medium, including HibernateA (Cat. # 10740-025) and Neurobasal (Cat. # 21103-049), B27 medium supplement (Cat. # 17504-010) were purchased from Life Technologies Inc. (Gaithersburg, MD). Glutamine (Cat. # G-3126) and poly-D-lysine (Cat. # P-6407) were purchased from Sigma Chemicals (St. Louis, MO). Papain (Cat. # 3119) was obtained form Worthington (Lakewood, NJ). Gentamicin (Cat. # 15710-015), FGF_2 (Cat. # 13256-029), and optiprep (Cat. # 103-0061) were purchased from Life Technologies Inc. (Gaithersburg, MD). Twelve mm glass coverslips (Cat. # P7-63-3029) were purchased from Carolina Biological (Burlington, NC). Falcon Brand 35 mm diameter dish (Cat. # 08-772-4A), Costar Brand Tissue Culture 24-well plates (Cat. # 07-200-84), 50 ml centrifuge tube (Cat. # 05-539-6), 15 ml centrifuge tube (Cat. # 05-539-5), and 9-inch Pasteur pipette (Cat. # 13-678-6B) were purchased from Fisher Scientific (Chicago, IL).

Neurite Outgrowth Assay: Human recombinant apoE3 (Cat. # P2003) and apoE4 (Cat. # P2004) were purchased from Panvera (Madison, WI). Lactoferrin (Cat. # L-4756) was obtained from Sigma Chemical (St. Louis, MO). Purified RAP was generously provided by Dr. Dudley Strickland (American Red Cross, Rockville, MD).

ApoE Quantification: The mouse anti-human apoE (Cat. # MAB 1062) used for immunoprecipitation, was purchased from Chemicon (Temecula, TA). Protein A-sepharose CL-4B (Cat. # P-3391) and BSA (Cat. # A-9418) were obtained from Sigma

Chemicals (St. Louis, MO). Goat anti-human apoE (Cat. # 178479) was purchased from Calbiochem (San Diego, CA). The HRP conjugated secondary antibody, rabbit anti-goat IgG (Cat. # AP106P) was obtained from Chemicon (Temecula, CA). All other materials used for apoE quantitation, including pre-cast 4-20% gradient gel (Cat. # FB3435), Millipore Immobilon-P Transfer Membrane (Cat. # IPVH00010), Pierce SuperSignal West Pico Chemiluminescent Substrate (Cat. # PI34080), Kodak BioMax Light-2 film (5 x 7', Cat. # 05-728-53), Tris (Cat. # BP154-1), Glycine (Cat. # BP381-1), SDS (Cat. # BP166-100), Tween 20 (Cat. # BP337-500) and sodium bromophenol blue (Cat. # BP114-25) were purchased from Fisher Scientific (Chicago, IL).

Immunocytochemistry: The mouse anti-neurofilament-200 (Cat. # N-5389), rabbit anti-galactocerebroside (Cat. # N-9152), and mouse anti-Glial Fibrillary Acidic Protein (GFAP) (Cat. # N-3893) were obtained from Sigma Chemicals (St. Louis, MO). The FITC-conjugated goat anti-mouse IgG-Fab_2 specific (Cat. # 115-095-006), FITC-conjugated AffiniPure goat anti-rabbit IgG (H+L) (Cat. # 111-095-144), Cy3 conjugated AffiniPure goat anti-mouse IgG (H+L) (Cat. # 111-165-146) and normal goat serum (Cat # 005-000-121) were purchased from Jackson ImmunoResearch (West Grove, PA). Paraformaldehyde (Cat. # O4042-500) was purchased from Fisher Scientific (Chicago, IL). Triton X-100 (Cat. # T-9284), n-Propyl gallate (Cat. # P-3130), and glycerol (Cat. # G-6279) were purchased from Sigma Chemicals (St. Louis, MO).

Methods:

Culture of AMC Neurons: For each experiment, a single male, 4 month old homozygous apoE KO or C57BL6/J mouse was anesthetized with sodium pentobarbital (80 mg/kg).

The entire cerebral cortex was dissected from the brain in 2 ml B27/HibernateA medium [B27/HibernateA with 0.5 mM glutamine] in a 35 mm diameter petridish at 4 °C. The cortex was sliced (0.5 mm) and transferred to a 50 ml tube containing 5 ml B27/HibernateA medium. After warming for 8 min at 30 °C, slices were digested with 6 ml of a 2 mg/ml papain solution in HibernateA for 30 min at 30 °C in a gyrating water bath to keep the slices suspended. Slices were transferred to 2 ml B27/HibernateA medium in a 15 ml tube. After 2 min at room temperature, slices were triturated 10 times with a siliconized 9-inch Pasteur pipette, and allowed to settle for 1 min. Approximately 2 ml of the supernatant was transferred to another tube, and the sediment was resuspended in 2 ml B27/HibernateA medium. The above step was repeated twice, and a total of 6 ml was collected. The resultant supernatant was subjected to density gradient centrifugation at 800 X g for 15 min. The Optiprep gradient was prepared in four 1 ml steps of 35, 25, 20 and 15% Optiprep in B27/HibernateA medium (v/v). The fraction containing the neurons was collected, and diluted in 5 ml B27/HibernateA medium. After centrifuging twice at 200 X g for 2 min, the cell pellets were resuspended in 3 ml B27/Neurobasal medium [B27/Neurobasal with 0.5 mM glutamine, no glutamate, 0.01 mg/ml gentamicin]. The cells in the suspension were counted in a hemacytometer, and plated in 40 to 50 µl aliquots on glass coverslips previously coated with 50 µg/ml poly-D-lysine in water. Coverslips were coated the night before the cell culture with 100 µl poly-D-lysine solution on each coverslip. On the day of the culture, coverslips were rinsed with distilled water and allowed to air-dry. Following 1h incubation in a humidified incubator at 37 °C and 5% CO_2, coverslips were transferred to a 24-well plate containing 0.4 ml B27/NeurobasalA medium. Coverslips were rinsed twice with

B27/HibernateA medium, and then 0.4 ml growth medium [B27/Neurobasal medium with 5 ng/ml FGF2] was added to each well and the plate was further incubated. After 4 days of incubation, neurite outgrowth was assessed as described below. For cultures longer than 4 days, half of the medium was replaced with B27/Neurobasal with 10 ng/ml FGF2 every four days.

Neurite Outgrowth: To assess neurite outgrowth, AMC cells were grown in growth medium (GM) alone or with other test reagents (human-apoE, inhibitors, etc.). Following 4 days of incubation, the length of the longest neurite (neurite extension) and total length of neurites (combined length) of each neuron were measured by using an inverted phase-contrast microscope. For each experiment, a minimum of 40 to 50 neurons were measured for each treatment condition. All experiments were repeated four times using different preparations of AMC neurons, and reagents. The data in individual experiments were presented as the mean ± standard error, and analyzed by an independent t-tests (α = 0.05).

In experiments that involved the effects of human apoE on neurite outgrowth, AMC cultures from apoE KO mice were prepared as previously described. After 24 hrs incubation, various concentrations of recombinant human apoE3 and recombinant human apoE4 were added directly to the medium. Commercial recombinant human apoE was purchased from Panvera and was dissolved in 0.7 M NH_4HCO_3. Before adding to the culture, apoE was dialyzed in 0.1 M NH_4HCO_3 overnight. On the fourth day of culture, neurite outgrowth was quantified.

In studies on the effects of inhibitors of lipoprotein receptors, AMC neurons from apoE KO mice were incubated for 24 hrs in growth medium prior to the addition of RAP

(5 μg/ml), or lactoferrin (10 μg/ml) to the medium. After one hour, recombinant human apoE3 (2.5 μg/ml) or recombinant human apoE4 (2.5 μg/ml) was added. The inhibitors were re-added to the medium every 24 hrs, assuming that the previously added inhibitors were totally consumed; following a 4-day incubation period, neurite outgrowth was assayed.

Medium Swapping Experiment: An AMC neuronal culture from control mice was prepared as described above. After a 24-hr period, AMC culture from apoE KO mice was prepared. After a 1-hr incubation, the culture medium from the apoE KO culture was removed and the cells were rinsed with B27/HibernateA medium. Conditioned medium from control cultures that were 24 hrs old, was centrifuged to remove suspended cells and cellular debris, was added to the KO culture. Every 24 hrs, the KO culture medium was replaced with medium from the control culture. On the fourth day of each culture, the neurite outgrowth was assayed.

ApoE Quantification: Medium from four-day-old control mice cultures was collected and centrifuged to eliminate suspended cells. ApoE was immunoprecipated by incubating the medium with 0.8 μg mouse anti-human apoE on ice for 1 hr. Following incubation, 50 μl of 10% Protein A-Sepharose CL-4B was added, and the medium was further incubated on ice for 1 hr on a shaker. The medium was centrifuged at 10,000 X g for 15 min at 4 °C, and the supernatant discarded. The pellet was boiled for 5 min in 2X Lammeli sample buffer (6.25 ml 4X Tris/SDS [pH 6.8], 5 ml glycerol, 1g SDS, 0.5 ml 2-mercaptoethanol, bromophenol, 13.25 ml dH_2O) and electrophoresed on 4-20% polyacrylamide gradient gels in an EC120 Mini gel vertical system. The samples were

run at 80 volts until separation began, and then at 140 volts until the dye front reached near the bottom of the gel. (Bellosta *et al.*, 1995).

Following electrophoresis, the gel was placed in transfer buffer (3.03 g Tris-base, 14.4 g glycine, 200 ml methanol, 800 ml dH_2O) on a shaker for 10 min. The transfer membrane, Millipore Immobilon-P Transfer Membrane, was first soaked in methanol for 5 sec and then washed in dH_2O for 5 min to remove excess methanol. The gel was placed on a presoaked filter paper in the holder and the transfer membrane was placed on top of the gel. The gel holder was placed in the buffer tank of Trans-blot Transfer Cell and the entire chamber was filled with transfer buffer. Proteins from the gel were transferred onto the membrane by passing 100 volts for an hour.

After transferring, blots were blocked in TBS buffer [20 mM Tris-HCl, 150 mM NaCl, pH 7.5] with 1% BSA at 4 °C overnight or at room temperature for 1 hr. Then blots were washed twice, 5 min each, with TBST buffer [TBS buffer with 0.05% Tween 20]. Blots were then incubated in goat anti-human apoE (1:5,000 dilution in TBST buffer) for 1 hr on a shaker at room temperature. The membrane was then washed 4 times, 10 minutes each, in TBST buffer. Blots were incubated in HRP conjugated rabbit anti-goat IgG (1:10,000 dilution in TBST buffer) for 1 hr on a shaker at room temp, washed 5 times, 10 min each, with TBST buffer, incubated with Pierce SuperSignal West Pico Chemiluminescent Substrate, and then exposed to Kodak BioMax Light-2 film. A 35 kDa band was visualized which is consistent with the published molecular weight of apoE.

All experiments were repeated at least three times to assure the reproducibility of the results. Bands were quantified by densitometry (Scion Image). Statistical analysis (unpaired, two tailed t-test) was performed by using Statview software.

Immunocytochemistry

a) Neurons: After four days of incubation, cells were rinsed with warm PBS (37 °C) and then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After rinsing twice with PBS, cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min. Cells were rinsed again with PBS, and then blocked with blocking solution [5% normal goat serum, 0.5% Triton X-100 in PBS] for 5 min. Cells were incubated overnight at 4 °C with mouse anti-neurofilament 200 (diluted 1:40) in the blocking solution. After rinsing four times with PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG-Fab_2 (diluted 1:200) in blocking solution at room temperature for 1 hr. Cells were washed four times in PBS, and the coverslips were mounted on glass slides in anti-photobleach [0.04g n-Propyl gallate, 6 ml glycerol, 1 ml 10xTBS, pH7.4, and bring the volume to 10 ml with dH_2O].

b) Oligodendrocytes: After a four day incubation period, cells were rinsed with warm PBS (37 °C) and then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After rinsing twice with PBS, cells were blocked with blocking solution [1% normal goat serum, 1% BSA in PBS] for 5 min. Cells were then incubated at room temperature for 1 hr with rabbit anti-galactocerebroside (diluted 1:50) in the blocking solution, rinsed four times with PBS, and incubated with FITC-conjugated AffiniPure goat anti-rabbit IgG (H+L) (diluted 1:200) in blocking solution at room temperature for 1

hr. Cells were then washed four times in PBS, and the coverslips were mounted on glass slides in anti-photobleach.

c) Astrocytes: After four days of incubation, cells were rinsed with warm PBS (37 °C) and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After rinsing twice with PBS, cells were blocked with blocking solution [5% normal goat serum, 0.05% Triton X-100 in PBS] for 5 min, and incubated at room temperature for 1 hr or 4 °C overnight with mouse monoclonal anti-Glial Fibrillary Acidic Protein (GFAP) (diluted 1:1200) in the blocking solution. After rinsing four times with PBS, cells were incubated with Cy3 conjugated AffiniPure goat anti-mouse IgG (H+L) (diluted 1:3000) in blocking solution at room temperature for 1 hr, then washed four times in PBS, and the coverslips were mounted on glass slides in anti-photobleach.

Results:

Characterization of AMC neuronal culture: As a first step towards studying the effects of apoE on adult neurons, I developed techniques for culturing adult mice neurons. The average total number of viable cells recovered from three preparations after 4 days in culture was 60.7 ± 3.3% (mean ± S.D.). Cell types in the culture were determined by immunofluorescent labeling of specific cell type markers; markers used are neurofilament 200 for neurons, galactocerebroside for oligodendrocytes, and GFAP for astrocytes. The majority of cells in the culture were neurofilament positive, representing ~ 70% of the cells. About 14% of the cells were stained with galactocerebroside. GFAP positive cells were present at low levels with an average of 13%.

The levels of apoE secreted into the medium by cells in the AMC culture was assessed by immunoblotting technique and quantitated by densitometry. The cells secreted 10.64 ± 0.62 ng/ml of apoE in 96 hrs.

Effects of deficiency of mouse apoE expression on neurite outgrowth: To evaluate whether lack of mouse apoE expression affects neurite outgrowth, I compared neurite extension and combined length of neurites in cultures of AMC neurons from apoE KO mice with cultures derived from the same age, sex, and strain matched wild-type mice. Neurite outgrowth was monitored at 96 hrs. AMC neurons from apoE KO mice had significantly shorter ($p<0.01$) neurite extension and combined length as compared with those in neurons from wild-type mice (Fig. 1 & 2). To directly test if the attenuated neurite outgrowth observed in apoE KO culture is due to the absence of apoE expression, I incubated the apoE KO neurons with conditioned medium from wild-type mice. Neurite outgrowth from apoE KO mice grown in conditioned medium from wild-type mice was

Figure 1. Effects of deficiency of mouse apoE expression on neurite extension of adult mice cortical neurons (mean ± pooled S.E., $\alpha < 0.05$). The AMC neurons from apoE KO mice had significantly shorter ($p<0.01$) neurite outgrowth as compared to control mice. There was no significant difference between apoE KO culture incubated with conditioned medium and control culture regarding to neurite extension.

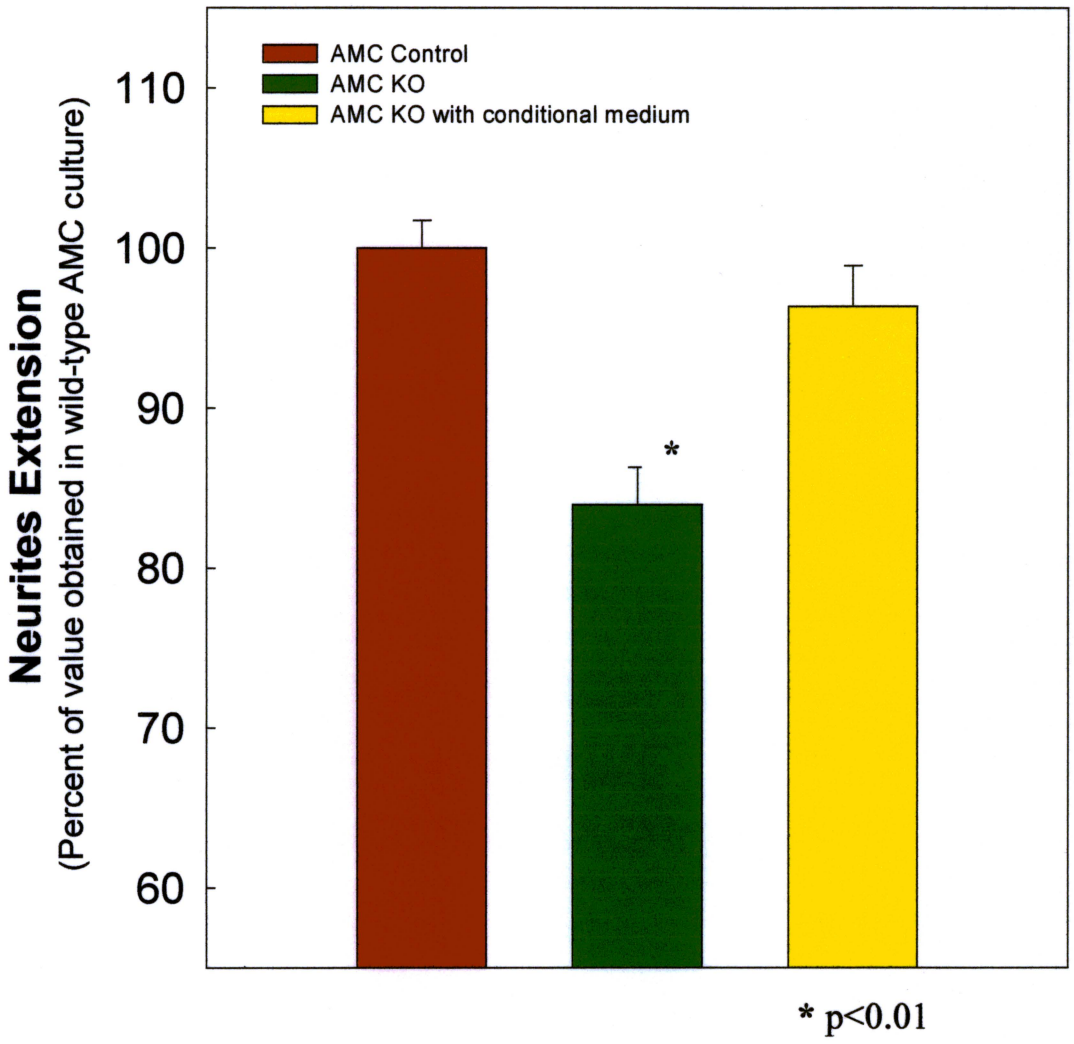
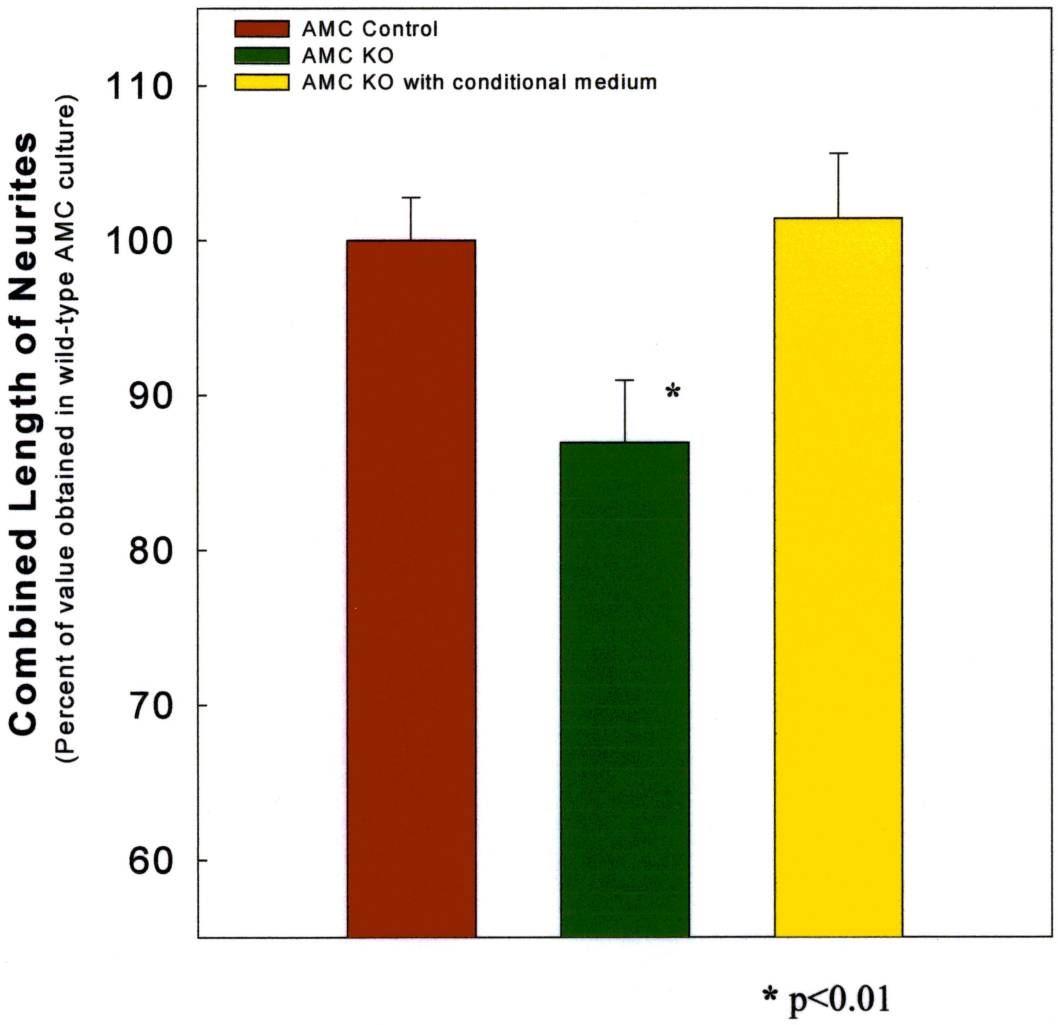


Figure 2. Effects of deficiency of mouse apoE expression on combined length of adult mice cortical neurons (mean ± pooled S.E., $\alpha < 0.05$). The combined length of the neurites of AMC neurons from KO mice was significantly less ($p<0.01$) than that in AMC neurons from control mice. In the presence of the control medium, the combined length of KO culture was similar to that of control culture.

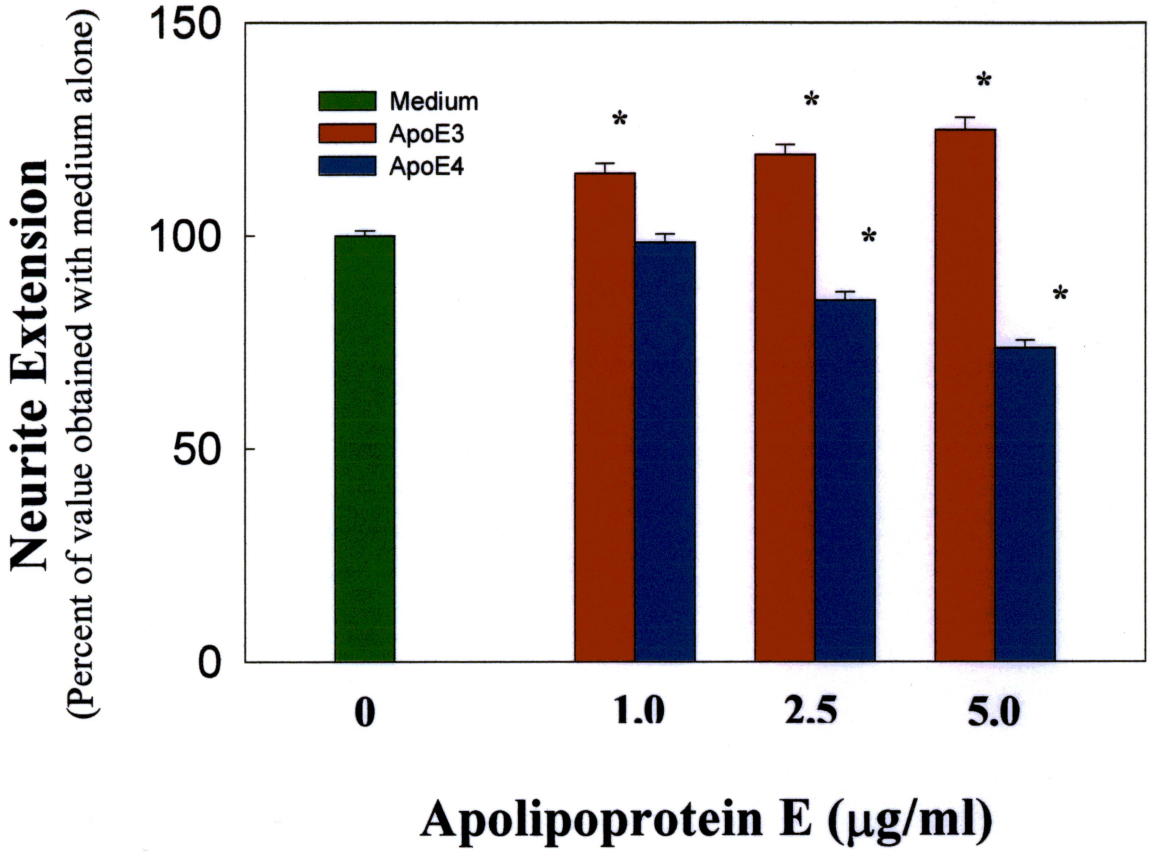


essentially similar to those in neurons from wild-type culture (Fig. 1 & 2). These data show that absence of apoE expression inhibits neurite outgrowth from AMC neurons.

Differential effects of human apoE3 and human apoE4 on neurite outgrowth: To determine whether human apoE isoforms affect neurite outgrowth from adult neurons, AMC culture from apoE KO mice were incubated in medium alone or in medium containing recombinant human apoE3 or human apoE4. Four days after treatment, neurons were scored for neurite outgrowth. Incubation of AMC culture with apoE3 significantly ($p<0.01$) increased both neurite extension and combined length (Fig. 3 & 4). In contrast to apoE3, addition of human apoE4 to the medium decreased ($p<0.01$) both neurite extension and combined length (Fig. 3 & 4), essentially stunting neurite outgrowth. These observations were confirmed when neurite outgrowth was quantified. Treatment of culture with apoE3 increased extension and combined length of neurites, whereas apoE4 significantly decreased both neurite extension and combined length. It is important to note that, addition of as little as 1 μg/ml of apoE4 increased neurite outgrowth from AMC neurons.

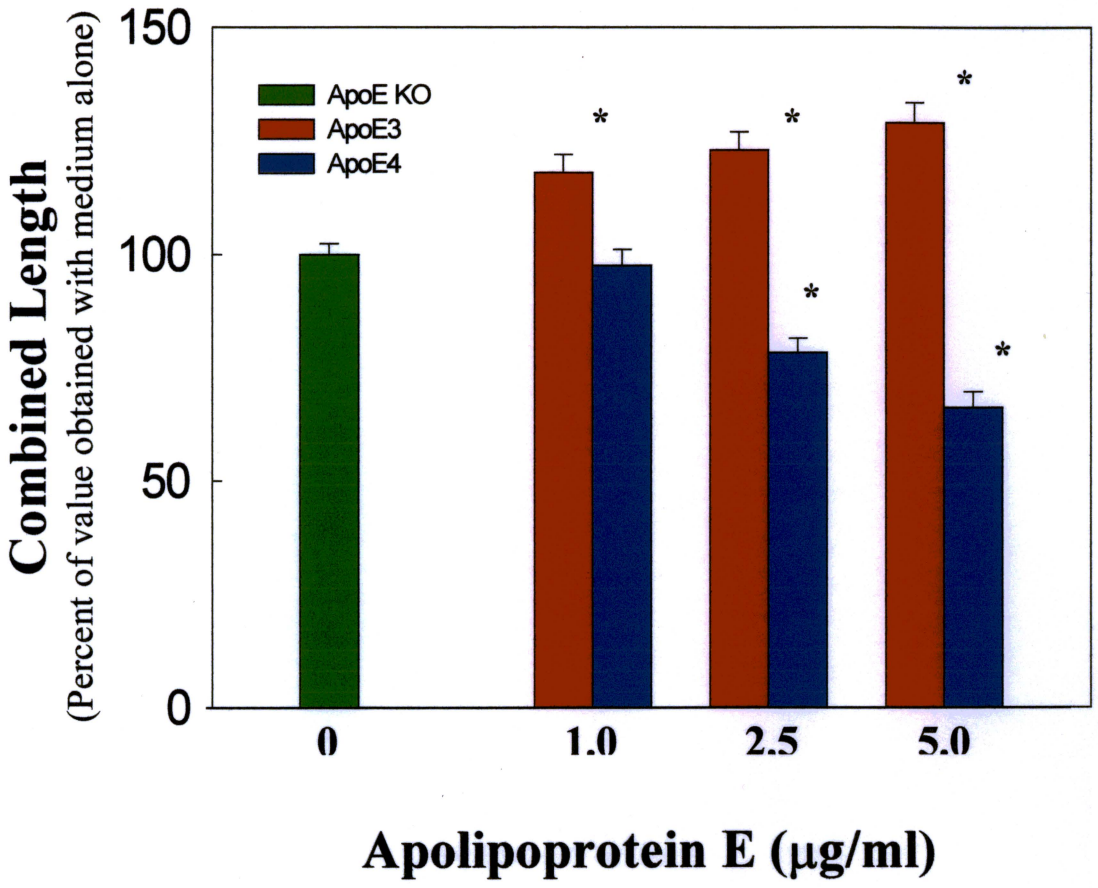
LRP pathway mediates the differential effects of apoE isoforms on neurite outgrowth: In previous studies our laboratory has shown that the effects of apoE on neurite growth are mediated by lipoprotein receptors. To determine which receptor was involved in mediating the differential effects of apoE3 and apoE4 on neurite outgrowth, I used inhibitors, lactoferrin and RAP to block the binding and internalization of apoE-containing lipoproteins by the LRP pathway. Both lactoferrin and RAP have been

Figure 3. Differential effects of human apoE3 and human apoE4 on neurite extension of adult mice cortical neurons (mean ± pooled S.E., $\alpha < 0.05$). Incubation of AMC KO culture with apoE3, significantly increased ($p<0.01$) neurite extension in a dose dependent fashion. Incubation of cells with apoE4 decreased ($p<0.01$) neurite extension in a dose dependent fashion.



* $p<0.01$

Figure 4. Differential effects of human apoE3 and human apoE4 on combined length of adult mice cortical neurons (mean ± pooled S.E., $\alpha < 0.05$). Incubation of AMC KO culture with apoE3, significantly increased ($p<0.01$) combined length in a dose dependent fashion while incubation of cells with apoE4 decreased ($p<0.01$) combined length in a dose dependent fashion.



* $p<0.01$

previously shown to abolish the binding of apoE-enriched lipoproteins to the LRP. As shown in Fig. 5 & 6, treatment of AMC culture with lactoferrin and RAP blocked the differential effects of the apoE isoforms on neurite outgrowth. In the presence of lactoferrin and RAP the stimulatory effects of apoE3, and the inhibitory effect of apoE4 were abolished, and both neurite extension and combined length of neurites from apoE-treated AMC neurons were similar to those observed in neurons grown in medium alone. These data show that inhibition of the interaction of apoE with the LRP prevents the isoform specific effects of human apoE on neurite outgrowth.

Figure 5. LRP pathway mediates the differential effects of apoE isoforms on neurite extension of adult mice cortical neurons (mean ± pooled S.E., $\alpha < 0.05$). In the presence of either lactoferrin or RAP, the stimulatory effect of apoE3, and the inhibitory effect of apoE4 were abolished, and neurite extension of apoE-treated KO neurons were similar to those observed in neurons grown in medium alone.

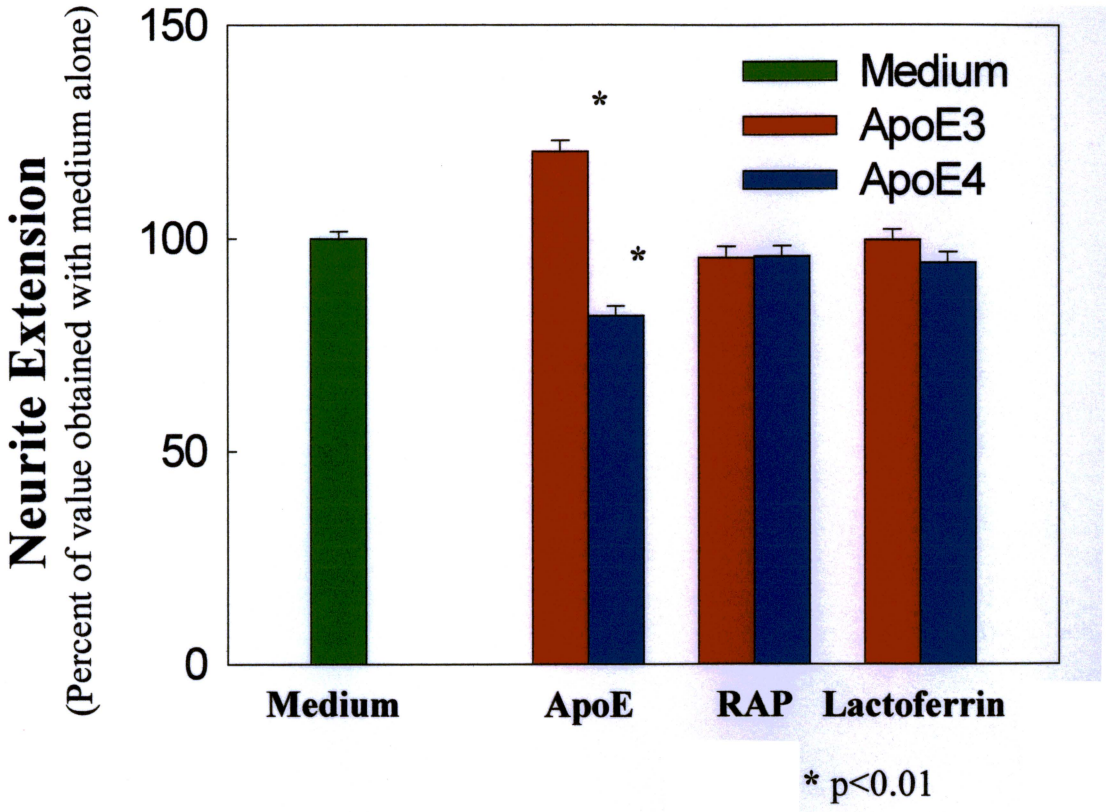
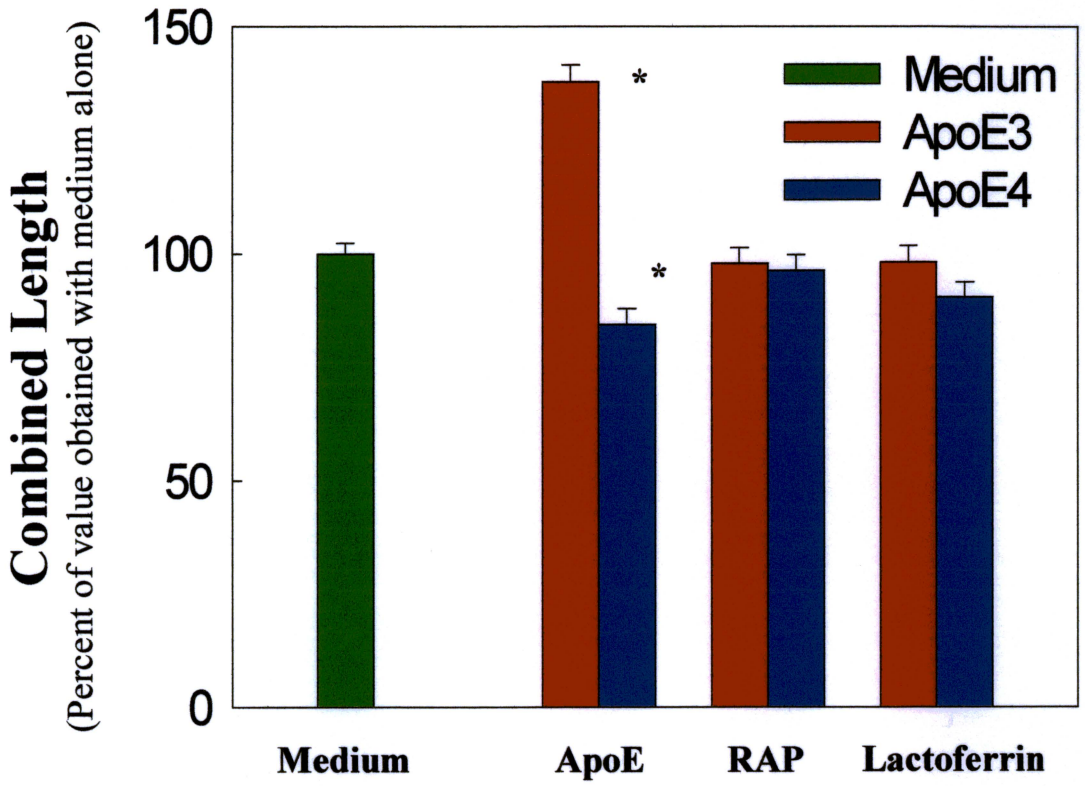


Figure 6. LRP pathway mediates the differential effects of apoE isoforms on combined length of adult mice cortical neurons (mean ± pooled S.E., $\alpha < 0.05$). In the presence of either lactoferrin or RAP, the stimulatory effect of apoE3, and the inhibitory effect of apoE4 were abolished, and combined length of apoE-treated KO neurons were similar to those observed in neurons grown in medium alone.



* $p<0.01$

Discussion:

Because of observations made over the past several years, the role of apoE in central nervous system (CNS) has drawn a lot of attention from researchers. ApoE messenger RNA has been found to be abundant in the brain and primarily synthesized and secreted by astrocytes and microglia macrophages (Elshourbagy *et al.*, 1985; Pitas *et al.*, 1987a). ApoE-containing lipoproteins are found in the cerebrospinal fluid and appear to play a major role in lipid transport in the CNS. It has also been found that apoE levels increase dramatically after peripheral nerve injury. Recently, apoE4 has been implicated to be associated with sporadic and late-onset familial AD (Poirier *et al.*, 1993; Beffert *et al.*, 1998). Numerous studies are now focusing on the mechanism whereby apoE leads to the pathogenesis of AD. One possible mechanism of apoE involvement in AD may be on processes involved in neurite development, given the well-known role of apoE in lipid transport. Several previous studies have demonstrated an isoform-specific effect of apoE on neurite outgrowth from both central nervous system- (Neuro-2a cells and GT1 trk 9 cells) and peripheral nervous system- (dorsal root ganglion cells) derived neurons (Nathan *et al.*, 1994; Bellosta *et al.*, 1995; Holtzman *et al.*, 1995; Nathan *et al.*, 1995). In the presence of lipoprotein, apoE3 enhances neurite outgrowth, whereas apoE4 inhibits neurite outgrowth. Although these studies have provided useful information, the relevance of these findings to adult neurons and AD is still not known.

The animal model used in this study, apoE KO mice, provides a unique opportunity to study the role of apoE in the CNS. In apoE KO mice, part of the apoE gene is replaced by a neomycin-resistance gene; this insertion disrupts the structure and function of apoE (Piedrahita *et al.*, 1992). ApoE KO mice have significant retardation in the acquisition of

developmental milestones, (forelimb placing behavior and cliff avoidance behavior) learning and memory impairments (Gozes *et al.*, 1997; Zhou *et al.*, 1998; Krzywkowski *et al.*, 1999), and altered stress responsiveness (Zhou *et al.*, 1998). It has also been found that apoE-deficient mice exhibit an impaired ability to recover from closed head injuries (Chen *et al.*, 1997). ApoE-deficient mice treated with either apoE3 or apoE4 show a significant improvement in their learning capacity in the Morris water maze compared to saline-infused apoE KO mice (Masliah *et al.*, 1997). Because apoE is a ligand for receptors that clear remnants of chylomicrons and VLDL, lack of apoE causes a high plasma cholesterol (5 fold) and severe occlusion of the coronary artery ostium in apoE-deficient mice (Zhang *et al.*, 1992).

Taken together, the apoE KO mouse system is an excellent model to study the role of apoE in the CNS and may shed light on the relationship between apoE and AD. By using this model, I have demonstrated that apoE isoforms exert differential effects on neurite outgrowth from adult mice cortical neurons. Incubation of AMC neurons with apoE3 increased both neurite extension and combined neurite length, whereas incubation with apoE4 decreased both extension and combined length, essentially stunting outgrowth. Furthermore, this study clearly demonstrates that the effects of apoE on neurite outgrowth are mediated via the LRP.

My studies provide evidence that nanogram quantities of endogenously produced mouse apoE facilitates neurite outgrowth. AMC neurons from apoE KO mice had significantly shorter neurite extensions and combined length of neurites as compared to those in AMC neurons from control mice. The decreased neurite outgrowth observed in apoE KO culture is not due to the property of the neurons, but is a result of the absence of

apoE production. The neurite outgrowth from apoE KO mice grown in conditioned medium from wild-type mice was essentially similar to those in neurons from wild-type culture. These results are consistent with other studies that showed impaired nerve recovery in apoE KO mice. Thus, endogenously secreted mouse apoE facilitates neurite outgrowth similar to human apoE3, even though mouse apoE is similar to human apoE4 in having arginine at positions 112 and 158. However, previous structural and functional studies on apoE have revealed that mouse apoE is "apoE3-like" because of the lack of arginine at position 61 which determines many of the isoform specific properties of apoE3 and apoE4, including the lipoprotein preferences.

I have demonstrated that the differential effects of apoE isoforms on neurite outgrowth seen in embryonic and neuronal cell lines also is observed in neurons derived from the adult brain. As little as 1 μg/ml recombinant human apoE3 present in KO culture can increase neurite extension. Addition of 5 μg/ml human apoE3 increased the length of the longest neurite by 25% and the combined length by 30% while the same amount of human apoE4 decreased the length of the longest neurite by almost 30% and the combined length by 35%. In previous studies (Nathan *et al.*, 1994; Bellosta *et al.*, 1995; Holtzman *et al.*, 1995; Nathan *et al.*, 1995), addition of a lipid source to the culture medium is required to elicit the differential effects of apoE3 and apoE4 on neurite outgrowth. In the AMC culture, however, purified apoE alone, in the absence of exogenous sources of lipid, led to the differential effects of apoE3 and apoE4 on neurite outgrowth. These results are not inconsistent since AMC culture is only a partially purified preparation and therefore contains cellular debris that could have served as a lipid source. In fact, a previous study (Nathan *et al.*, 1994) has shown that purified rabbit

apoE, in the absence of a source of lipid, reduced neurite outgrowth from a partially purified preparation of rabbit DRG neuron.

In previous studies it has been shown that the isoform-specific effects of apoE3 and apoE4 on neurite outgrowth appeared to require the interaction of apoE-containing lipoproteins with the LRP (Holtzman *et al.*, 1995). The role of the HSPG in the process is to sequester apoE-enriched lipoproteins. Consistent with these previous findings, my data also suggest that the LRP/HSPG pathway is important for the differential effects of apoE3 and apoE4 on neurite outgrowth in AMC neurons. In the presence of lactoferrin (lactoferin blocks the binding of lipoprotein to both HSPG and the LRP) or RAP, the differential effects on neurite outgrowth caused by apoE3 and apoE4 in the KO AMC culture were abolished.

The precise mechanism by which apoE leads to the development of AD is unclear. However, based on my results, I have developed a model that might help to explain the potential role of apoE in the pathogenesis of AD. The model proposes that AD is an imbalance between neuronal injury and efficient repair. In this model, neurons that are debilitated by age are more susceptible to neurotoxic agents (e.g., amyloid peptide, free radicals). The damage inflicted by these injurious agents signal the need for repair or remodeling of the neurons and neurites. At this stage, apoE3 in the brain may support the repair process, whereas apoE4 being produced in the aging brain would not support repair mechanism. Thus apoE4 would serve as a susceptibility factor for the development of neurodegenerative diseases like Alzheimer's disease.

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