Effects of E2 on ApoE Secretion and Neurite Outgrowth in Cultured Adult Mouse Cortical Neurons

Fei Shen
Eastern Illinois University

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Effects of E2 on ApoE Secretion and Neurite Outgrowth in Cultured Adult Mouse Cortical Neurons

(TITLE)

BY

Fei Shen

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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2002
YEAR

I HEREBY RECOMMEND THAT THIS THESIS BE ACCEPTED AS FULFILLING THIS PART OF THE GRADUATE DEGREE CITED ABOVE

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Abstract:

Estrogen replacement therapy appears to delay the onset of Alzheimer’s disease (AD). The mechanism whereby estrogen prevents the pathogenesis of AD is unknown. In the present study, I examined the effects of 17-β-estradiol (E2) on neurite outgrowth from adult mice cortical neurons (AMC) in culture. I found that E2 increases apoE secretion and neurite outgrowth in AMC neurons from wild type mice in a dose dependent fashion. The neurite outgrowth promoting effect of E2 was not observed in AMC neurons derived from age-, sex-, and stain-matched apoE deficient/apoE gene knockout (apoE KO) mice. Furthermore, E2 has isoform specific effects on neurite outgrowth in the presence of purified recombinant human apoE. The presence of human apoE2 or apoE3 greatly augmented E2 effects on promoting neurite outgrowth, whereas the presence of apoE4 had no significant effect. Consistent with these findings, E2 had differential effects on neurite outgrowth from AMC neurons derived from transgenic mice expressing human apoE isoforms. Incubation of AMC neurons from apoE3 transgenic mice with E2 significantly increased neurite outgrowth, whereas incubation of AMC neurons from apoE4 transgenic mice with E2 had no significant effect. In summary, my data suggest that apoE isoforms play a critical role in mediating the neurite outgrowth promoting effect of E2.
1. Introduction:

Alzheimer's Disease (AD) is an irreversible, progressive brain disorder that occurs gradually and results in memory loss, unusual behavior, personality changes, and a decline in thinking ability. AD 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).

Apolipoprotein E (apoE) is a 299 amino acid component of lipoproteins with a molecular weight of 37-kDa that mediates endocytotic lipid uptake by cells via several lipoprotein receptors. ApoE plays a vital role in the regulation of lipoprotein metabolism and in the control of lipid transport and lipid redistribution among target tissues and cells (Mahley, 1988; Weisgraber, 1994). Lipid transport and redistribution is regulated by apoE via interaction with lipoprotein receptors (Mahley, 1988). Cellular uptake and degradation of the lipoproteins is initiated by receptor-lipoprotein binding. The lipid becomes available for utilization in the regulation of intracellular cholesterol metabolism.
ApoE, therefore, serves as a ligand for the receptor-mediated clearance of lipoproteins from the plasma (Rall et al., 1982).

ApoE is encoded by a single polymorphic gene, which contains four exons on chromosome 19 in humans (Mahley, 1988; Rall et al., 1982; Das et al., 1985; Paik et al., 1985). The three major allele isoforms for the apoE gene are e2, e3, and e4, the frequencies of these three alleles in the general population are 8%, 77%, and 15%, respectively (Gooch and Stennet., 1996; Mahley 1994). The molecular basis of this polymorphism of the apoE gene results from cysteine-arginine interchanges at two positions in the apoE protein (Weisgraber, 1994). These single amino acid substitutions are found at residues 112 and 158 (Rall et al., 1982). The most common isoform, apoE3, contains cysteine at residue 112 and arginine at position 158. ApoE2 has cysteine at both positions and apoE4 contains arginine at both positions (Weisgraber, 1994). 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 (Gooch and Stennet., 1996).

It is believed apoE functions as a lipid carrier molecule in membrane remodeling in the response to peripheral and central nerve damage, possibly to scavenge cholesterol from cellular debris for use in axonal regeneration and remyelination (Ignatius et al., 1986; Mahley, 1988; Poirier, 1996). In humans the brain is second only to the liver in apoE production (Beffert et al., 1998; Elshourbagy et al., 1985). Transcripts for apoE are distributed throughout all regions of the brain, and have been localized to astrocytes and microglia by in situ hybridization. In the central nervous system (CNS), apoE is synthesized and secreted primarily by glial cells (astrocytes, microglia and to a lesser
extent, by macrophages) (Elshourbagy et al., 1985; Pitas et al., 1987) but not by neurons. ApoE serves as a ligand for the low-density lipoprotein receptor (LDLR) in primary cultures of rat brain astrocytes (Beffert et al., 1998). In cerebrospinal fluid, apoE is found associated with high-density lipoprotein (HDL) particles and appear to play a major role in lipid transport in the CNS. 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 injury (Boyles et al., 1990; Boyles et al., 1989; Ignatius et al., 1986; Leblanc et al., 1990).

Increased apoE immunoreactivity is present in the brains of patients with such neurological disorders as Down’s syndrome, Creutzfeld-Jacob disease, and Alzheimer’s disease (AD) (Namba et al., 1991). It has been demonstrated that expression of apoE increases following optic nerve injury, but absolute levels of apoE do not increase (Ignatius et al., 1986). ApoE mRNA is increased in the brains of AD patients and in the response to injury in both CNS (Boyles et al., 1989) and peripheral nerve system (Snipes et al., 1986).

In culture from fetal dorsal root ganglion cultures, incubation with β-very low-density lipoprotein (β-VLDL) particles, which are rich in both apoE and cholesterol, showed increased neurite outgrowth and branching (Handelmann et al., 1992). These results have been the basis for proposing that apoE-containing lipoproteins are involved in the mobilization and redistribution of lipids in the repair and maintenance of myelin and axonal membrane following peripheral nerve injury (Beffert et al., 1998). Based on these observations, it has also been proposed that apoE is involved in neurodegenerative processes by isoform-specific effects on cytoskeletal stability and neurite outgrowth.
(Mahley et al., 1994; Weisgraber and Mahley, 1996). In vitro studies with dorsal root ganglion neurons have shown that addition of apoE3 to a culture stimulated neurite outgrowth whereas apoE4 decreased neurite extension (Nathan et al., 1994). Former studies from our laboratory have shown that addition of apoE3 to adult mouse cortical neuron cultures significantly increased neurite outgrowth. These data imply that apoE is important for peripheral nerve regeneration (Mahley, 1988). The data from apoE knockout (apoE KO) mice, however, does not support this hypothesis. Regeneration of nerves in both control mice and apoE KO mice were morphologically identical at two and four weeks following sciatic nerve crush (Popko et al., 1993; Goodrum, 1995). This suggests that other apolipoproteins in the PNS may substitute for apoE when it is absent. Therefore, the specific role of apoE and its importance in the PNS remains unclear.

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 the 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 are 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.

In addition to apoE genotype, it seems that estrogen plays an important protective role in the development of AD. Estrogen is a hormone dominant in the female reproductive system. Estrogen can improve blood circulation in the brain as well as stimulate nerve cell growth. It has also been shown to increase levels of acetylcholine, and it impedes the deposition of amyloid (Inestrosa et al., 1998). Women are statistically more susceptible to AD which leads researchers to believe that hormones may also play a crucial role in the disease process. A plausible explanation for the increased occurrence of AD in women is due to the loss of ovarian steroids in the menopause stage. The normal age of menopause is 54 years and the average life span of women is 78 years. This indicates that most women spend almost one-third of their life in an estrogen-deprived state. There is recent evidence that postmenopausal estrogen replacement therapy (ERT) is inversely correlated, both in dose and duration of therapy, with the incidence of AD (Phillips et al., 1992). Preliminary trials indicate a positive effect of ERT on cognition in both cognitively impaired and normal postmenopausal women (Sherwin 1988). Additional studies have shown that estrogen serves a normal maintenance role in the same regions of the brain that are most effected in AD. Estrogen treatment in experimental animals promotes recovery from neurological damage. Estrogen administration increases glial cell markers and apoE in most of these animal models (Simpkins et al., 1994).
Recently studies have suggested that estrogen's protective effects are through its action as a trophic factor for cholinergic neurons, a modulator for the expression of apoE in the brain, an antioxidant compound decreasing the neuronal damage caused by oxidative stress (Inestrosa et al., 1998). In mixed glial cultures that contain both astrocytes and microglia, 17-β-estradiol (E2) treatment induced two-fold increases in apoE mRNA over untreated controls and the largest increase is seen at an estrogen concentration of 0.1 nM, corresponding to physiological levels of circulating estrogen during proestrus (0.05 nM) and pregnancy (0.5 nM) (Stone et al., 1997). Previous studies have shown that estrogen treatment significantly increased the neurite outgrowth of acetylcholinesterase-positive fibers from embryonic basal-forebrain tissues transplanted into the anterior chamber of the eye (Honjo et al., 1992), indicating that estrogen may have a direct trophic effect upon basal-forebrain cholinergic neurons. The cholinergic neurons located in the basal forebrain are particulary susceptible to degeneration in AD and have been correlated in age and disease-related cognitive decline (Whitehouse et al., 1981). These results indicate that estrogen could have positive effects on memory and learning processes (Gibbs 1997).

The way in which estrogen interacts with apoE and neurons is not completely known. The mechanism for this process would prove to be quite important for developing novel therapies for Alzheimer's disease. The primary aim of my study was to determine the pathway by which 17-β-estradiol (E2) increases neurite outgrowth from AMC neurons. In this thesis, I demonstrate the following specific questions: 1) E2 effect on apoE secretion and neurite outgrowth in AMC neurons from wild type mice. 2) E2 effect on neurite outgrowth in AMC neurons derived from age-, sex-, and stain-matched
apoE deficient/apoE gene knockout (apoE KO) mice. 3) Does E2 have isoform specific effects on neurite outgrowth in the presence of purified recombinant human apoE? 4) E2 effect on neurite outgrowth from AMC neurons derived from transgenic mice expressing human apoE isoforms.
2. Materials and Methods

2.1. Materials

2.1.1. Adult mouse cortical culture

Transgenic human apoE3, transgenic human apoE4 mice, homozygous apoE KO mice (C57BL/6-Apo<sup>tmUnc</sup>, Cat. # 002052) bred 10 generations onto the C57BL/6 background and control mice (C57BL/6, Cat. # 000664) were obtained from Jackson Laboratory (Bar Harbor, MA). Cell culture medium, including Hibernate A (Cat. # 10740-025), Neurobasal (Cat. # 21103-049), and 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 from Worthington (Lakewood, NJ). Gentamicin (Cat. # 15710-015), FGF2 (Cat. # 13256-029), and Optiprep (Cat. # 103-0061) were from Life Technologies Inc., (Gaithersburg, MD). Glass cover slips (Cat. # P7-63-3029) were purchased from Carolina Biological (Burlington, NC). Falcon Brand 35 mm diameter dishes (Cat. # 08-772-4A), Costar Brand Tissue Culture 24-well plates (Cat. # 07-200-84), 50 ml tube (Cat. # 05-539-6), 15 ml centrifuge tubes (Cat. # 05-539-5), and 9-inch pipettes (Cat. # 13-678-6B) were purchased from Fisher Scientific (Chicago, IL).

2.1.2. Neurite outgrowth assay

Human recombinant apoE3 (Cat. # P2003) and apoE4 (Cat. # P2004) were purchased from Panvera (Madison, WI), and dialyzed overnight in 0.1M ammonium bicarbonate. 17-β-estradiol (Cat. # E-3229) was purchased from Sigma (St. Louis, MO) and was dissolved in 95% ethanol.

2.1.3. ApoE quantification
The monoclonal anti-apoE (3H1) used for immunoprecipitation was obtained from the University of Ottawa, Heart Institute. HRP conjugated secondary antibody, and rabbit anti-goat IgG (Cat. # AP106P) was obtained from Chemicon (Temecula, CA). 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). All other materials used for apoE quantification, including pre-cast 4-20% gradient gels (Cat. # FB3435), Millipore Immobilon-P Transfer Membranes (Cat. # IPVH00010), Pierce SuperSignal West Pico Chemiluminescent Substrate (Cat. # PI34080), Kodak BioMax Light-2 film (5*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. # BP-114-25) were purchased from Fisher Scientific (Chicago, IL).

2.1.4. Immunocytochemistry

The mouse anti-neurofilament-70 (Cat. #N-5388), mouse anti-Glial Fibrillary Acidic Protein (GFAP) (Cat. # N-3893), and FITC-conjugated lectin from Bandeiraea simplicifolia (BSL-1) were obtained from Sigma Chemicals (St. Louis, MO). The FITC-conjugated goat anti-mouse IgG-Fab2 specific (Cat. # 115-095-006) 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 (St. Louis, MO). 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).
2.2. Methods

2.2.1 Adult mouse cortical neuronal culture

For each experiment, a single female, 4 month old mouse was anesthetized with sodium pentobarbital (80 mg/kg). The entire cerebral cortex was dissected from the rest of the brain in 2 ml B27/Hibernate A medium [B27/Hibernate A with 0.5 mM glutamine] in a 35 mm diameter Petri dish placed at 4°C. The cortex was sliced (0.5 mm thickness) and transferred to a 50 ml tube containing 5 ml B27/Hibernate A medium. After warming for 8 min at 30°C, slices were digested with 6 ml of a 2 mg/ml papain solution in Hibernate A for 30 min at 30°C in a gyrating water bath to keep the slices suspended. Slices were transferred to 2 ml B27/Hibernate A 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 resuspended in 2 ml B27/Hibernate A medium. The above step was repeated twice, and a total of 6 ml collected. The resultant supernatant was subjected to density gradient centrifugation at 800g for 15 min. The density gradient was prepared in four 1 ml steps of 35, 25, 20 and 15% Optiprep in B27/Hibernate A medium (v/v). The brain fraction containing the neurons was collected, and diluted in 5 ml B27/Hibernate A medium. After centrifuging twice at 200g for 2 min, the cell pellets were resuspended in 3 ml B27/Neurobasal A medium [B27/Neurobasal A with 0.5 mM glutamine, no glutamate, 0.01 mg/ml gentamicin]. The cells in the suspension were counted in a hemacytometer, and 40,000 cells were plated in 50 µl aliquots on glass cover slips (12 mm diameter) previously coated overnight with 100 µl of 50 µg/ml poly-D-lysine in water. Following 1 hr incubation in a humidified incubator at 37 °C and 5% CO₂,
cover slips were transferred to a 24-well plate containing 0.4 ml B27/Neurobasal A medium. Cover slips were rinsed twice with B27/Hibemate A medium, and then 0.4 ml growth medium [B27/Neurobasal A medium with 5 ng/ml FGF2] was added to each well and the plate was further incubated. Neurite outgrowth, and viability assays were performed after 4 days of incubation, as described below. For culture periods greater than 4 days, half of the medium was replaced with B27/Neurobasal A with 10 ng/ml FGF2 every four days.

2.2.2. Neurite Outgrowth Assay

To assess neurite outgrowth, neurons were grown in growth medium (GM) alone or with other test reagents (E2, 5µg/ml human-apoE, etc). Following 3 days of incubation, the length of the longest neurite (neurite extension) and total length of neurites (combined length) of each neuron were measured using an inverted phase-contrast microscope. For each experiment, a minimum of 50 neurons were measured for each treatment condition.

In experiments that involved the effects of estrogen and human apoE isoforms on neurite outgrowth, neurons from adult mice were prepared as described above. After 24 hours incubation, human apoE isoforms or various concentrations of estrogen were added directly to the medium and continued incubating for another 48 hours. On the third day of culture, neurite outgrowth was quantified as described.

2.2.3. ApoE quantification

Two ml of medium from three-day-old cultures were collected and centrifuged to eliminate suspended cells. ApoE was immunoprecipitated by incubating the medium with 2 µg of monoclonal anti-apoE (3H1) 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 10,000g for 15 min at 4 °C, and the supernatant discarded. The pellet was boiled for 5 min in 2X Lammeli sample buffer and electrophoresed on 4-20% SDS-polyacrylamide gradient gels in an EC120 Mini gel vertical system. The samples were run at 80 V until separation began, and then at 140 V until the dye front neared 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 dH₂O) on a shaker for 10 min. The transfer membrane, Millipore Immobilon-P Transfer Membrane, was first soaked in methanol for 5 sec then washed in dH₂O for 5 min to remove excess methanol. The proteins in the gel were transferred to the Immobilon-P membrane using a Bio-Rad Trans-blot Transfer Cell following manufacturer's protocol.

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 hour on a shaker at room temperature. The membrane was then washed 4 times (10 min 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 temperature, 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. Bands were quantified by densitometry (Scion Image).

2.2.4. Immunocytochemistry

Cells from 4-day old cultures were rinsed with warm PBS (37°C) and 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, 0.05% Triton X-100 in PBS] for 1 hr. Cells were incubated overnight at 4°C with mouse anti-neurofilament 200 (1:40), or mouse anti-GFAP (1:1,200) in the blocking solution. After rinsing four times with PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG-Fab$_2$ (1:200) in blocking solution for 60 min. Cells were washed four times in PBS, slips were mounted in anti-photobleach medium (0.85 M n-propyl gallate, 60% glycerol in TBS). Immunoreactive cells were counted and photographed on an Olympus BX50 microscope with appropriate fluorescence excitation filters. Controls without primary antibody were negative.

For BSL1 labeling of microglia, cells were fixed as described above, rinsed with warm PBS, and incubated with fluorescein conjugated BSL1 (1:100 dilution) for 15 min at room temperature. Following incubation the cells were rinsed with PBS, and fixed with 4% paraformaldehyde in PBS for 5 min. After rinsing in PBS, slips were mounted and photographed as described above.

**2.2.5 Statistical Analysis**

All experiments were repeated four times using different preparations of adult mouse cortical neurons, and reagents. The data in individual experiments were presented as the mean ± standard error, and statistical analysis (unpaired, two tailed t-test) was performed using Statview software.
3. Results:

3.1. Characterization of adult mouse cortical neuronal culture

I modified techniques for the in vitro culture of neurons from adult mice as previously described (Brewer, 1997). The total recovery of viable cells after 3 days in culture averaged 65-70% of the cells plated. Fig. 1 shows the results for immunofluorescent labeling of cell types in the culture. The majority of the cells in culture were neurofilament positive, representing ~ 70% of the cells (Fig. 1A). GFAP positive cells comprised an average of 13%, while BSL1 stained cells represented about 14% of the total cells in culture. These three markers identified an average of 97% of the viable cells present in a three-day old culture. Representative morphologies of immunostained cells are shown in Fig. 1B. Neurofilament 200 immunoreactive cells displayed several long processes with branches. GFAP positive cells had a smaller number of processes with fewer branches. Microglial cells were flat and round with very few short processes. The concentration of apoE in the medium of a 3-day-old WT culture, as determined by quantitative immunoblotting, was 25-37 ng/ml. Medium from apoE KO culture had no detectable apoE.

3.2. Effects of E2 on neurite outgrowth from adult mouse cortical neurons derived from wide type mice

Cultures of AMC neurons from wild type mice were treated with 0.1 pM, 1.0 pM, 0.1 nM, 1.0 nM and 10 nM of E2, and neurite extension and combined length of neurites in cultures were quantified. E2 concentrations were added at 24 hours and neurite outgrowth was monitored at 72 hours. AMC neurons treated 1 pM, 0.1 nM and 1 nM E2 had significantly longer (p<0.01) neurite extension and combined length as compared to
Fig 1. Immunofluorescent labeling of cell types in the adult mouse cortical culture (A) Cortical neuronal cultures were grown for 4 days, fixed and stained for NF200, GFAP, and BSL1 as described in Methods. For each antigen, three coverslips were immunostained, and immunopositive cell in 12 fields/slip were counted and expressed as a percentage of total viable cells. (B) Representative morphologies of immunostained cells in cortical culture.
Fig. 2A. Effects of E2 on Neurite Outgrowth from Adult Mouse Cortical Neurons. Phase contrast photographs of representative neurons from wild type mice were incubated for 3 days in medium alone and in medium containing 0.1nM E2. Scale bar = 30μm.
Fig. 2B. Quantification of the Effects of E2 of the Dose Response on Neurite Extension (a) and Combined Length (b) from Adult Mouse Cortical Neurons. Neurite extension and combined length measured for 120 neurons from each group as described under Methods. Data are mean ±SEM (n=120).
neurons grown in medium alone (Fig. 2.).

3.3 Effects of E2 on apoE secretion from adult mouse cortical neurons derived from wild type mice

AMC cultures from wild type mice were incubated with varying concentration of estrogen as described above. The secreted apoE in the medium was quantified by immunoprecipitation followed by immunoblotting. AMC cultures treated with 1.0 pM, 0.1 nM and 1.0 nM E2 secreted significantly more apoE concentration (p<0.01) than that in culture grown in medium alone (Fig. 3.).

3.4. Time course of E2 on apoE secretion from adult mouse cortical neurons

The time course of apoE secretion in AMC culture was evaluated by incubating the culture with 0.1 nM E2 for 0, 0.1, 0.5, 1.0, 4.0, 16.0, 36.0, 48.0 hours. Following incubation, the medium was collected and secreted apoE was quantified by immunoprecipitation followed by immunoblotting. ApoE secretion significantly increased (p<0.01) after 4 hr, and plateaued after 16 hr incubation (Fig. 4.). These data suggest a time-dependent increase in apoE secretion as a function of E2 incubation.

3.5. Effects of E2 on adult mouse cortical neurons from apoE deficient (apoE KO) mice

To examine the impact of apoE deficiency on E2 effects on neurite outgrowth, I incubated AMC neurons from apoE KO and wide type control mice (C57BL/6) with the physiological concentration (0.1 nM) of E2. Following 3 days of incubation neurite outgrowth was monitored. Results from this study demonstrated that E2 had no significant effect on neurite outgrowth from AMC neurons derived from apoE KO mice (Fig. 5).
Fig. 3. Effects of E2 on ApoE Secretion from Adult Mouse Cortical Neurons. AMC culture from wild type mice was incubated for 3 days in medium alone or in medium containing various concentrations of E2. Medium was collected and ApoE in 2ml of medium was immunoprecipitated and immunoblotted as described in methods section. A. Photograph of a representative immunoblot. B. Densitometric scanning of immunoblot obtained from three independent experiments. Data are presented as its mean ± SEM (n=3).
Fig. 4. Time Course of E2 on ApoE Secretion from AMC Neurons. AMC culture from wide type mice was incubated with 0.1nM E2 for the indicated hours. Following incubation the medium was collected and apoE in 2ml medium was immunoprecipitated and immunoblotted as described in Method section. A. Photograph of a representative immunoblot. B. Densitometric scanning of immunoblot obtained from three independent experiments. Data are presented as its mean ± SEM.
Fig. 5A. Effects of E2 on Neurite Outgrowth from ApoE KO AMC Neurons. Phase contrast photograph of representation neurons from ApoE KO mice grown for 3 days in medium alone, in medium containing 0.1nM ethanol or 0.1nM E2 dissolved ethanol.
Fig. 5B. Quantification of the Effects of E2 on Neurite Extension (a) and Combined Length (b) from apoE KO AMC Neurons. Neurite extension and combined length for 120 neurons from each group as described under Method. Data are mean ±SEM (n=120).
Fig. 6A. Combined Effects of E2 and Recombinant Human ApoE on Neurite Outgrowth from ApoE KO AMC Neurons. Phase contrast photograph of representation neurons from apoE KO mice grown in 3 days in medium alone, in medium containing 0.1nM ethanol, 0.1nM E2, 5 µg/ml purified human apoE2, 5 µg/ml purified human apoE3 and 5 µg/ml purified human apoE4.
Fig. 6B. Quantification of the Effects of E2 and Recombinant Human ApoE on Neurite Extension (a) and Combined Length (b) from apoE KO AMC Neurons. Neurite extension and combined length for 120 neurons from each group as described in method. Data are mean ±SEM (n=120).
3.6. Effects of E2 on neurite outgrowth from adult mouse cortical neurons derived from apoE KO mice in presence of recombinant human apoE isoforms

Previous studies from our laboratory have shown that apoE isoforms can modulate neurite outgrowth from AMC neurons. In the present study we examined if apoE isoforms can alter neurite outgrowth promoting effect of E2. As shown in Fig. 6, incubation of AMC culture with human apoE2 and apoE3 significantly (p<0.01) increased both neurite extension and combined length. In contrast to apoE2 and apoE3, addition of human apoE4 did not essentially affect (p>0.05) neurite outgrowth. Furthermore, the presence of human apoE2 or apoE3 greatly augmented E2 effects (p<0.01) on promoting neurite outgrowth, whereas the presence of apoE4 had no significant effect (p>0.05).

3.7. Effects of E2 on adult mouse cortical neurons derived from human apoE transgenic mice

I next examined the effect of E2 on neurite outgrowth from AMC neurons derived from transgenic mice expressing human apoE3 or human apoE4. Consistent with previous studies AMC neurons from apoE3 mice grown in medium alone had greater neurite extension and combined length as compared to those neurons derived from apoE4 transgenic mice (Holtzman et al., 1995). Furthermore, incubation of AMC neurons from apoE3 transgenic mice with E2 significantly increased neurite outgrowth, whereas incubation of AMC neurons from apoE4 transgenic mice with E2 had no significant effect (Fig. 7.).

Western blot analysis of human apoE secretion revealed that both apoE3 and apoE4 AMC cultures secrete similar amounts of apoE. E2 treatment upregulated apoE
Fig. 7A. Effects of E2 on Neurite Outgrowth from AMC Neurons Derived from Transgenic Mice (Tg) Expressing Human ApoE3 or Human ApoE4. Phase contrast photograph of representation neurons grown on medium alone or in medium containing 0.1nM E2.
Fig. 7B. Quantification of the Effects of E2 on Neurite Extension (a) and Combined Length (b) from AMC Neurons Derived from Transgenic Mice Expressing Human ApoE3 or Human ApoE4. Neurite extension Neurite extension and combined length for 120 neurons from each group as described under Method. Data are mean ±SEM (n=120).
Fig. 8. Effect of E2 on ApoE Secretion from AMC Neurons Derived from Transgenic Mice (Tg). AMC culture from Tg-apoE3 and Tg-apoE4 was incubated with medium alone or in medium containing 0.1nM E2. Following 3 days of incubation, the medium was collected and apoE in 2ml of medium was immunoprecipitated and immunoblotted as described in Method section. A. Photograph of a representative immunoblot. B. Densitometric scanning of immunoblot obtained from three independent experiments. Data are presented as its mean ± SEM (n=3).
secretion in both apoE3 and apoE4 cultures, similar to that in wild type culture. There was no significant difference in the amount of apoE secreted by AMC cultures from apoE3 and apoE4 mice treated with E2 (Fig. 8.).
4. Discussion:

E2 replacement therapy appears to delay the onset of Alzheimer’s disease, but the mechanisms for this action are not known. E2 loss is not an issue for males, because they produce endogenous E2 throughout their lives. ApoE is of special importance in the nervous system. ApoE with a source of cholesterol promote marked neurite extension in cultured dorsal root ganglion cells. ApoE is involved in the response to neural injury (Boyles et al., 1990; Poirier et al., 1991; Poirier, 1994), maintainance of dendritic complexes (Masliah et al., 1995) and neuronal remodeling in vitro (Nathan et al., 1994; Fafan et al., 1996) and in AD (Arendt et al., 1997).

Previous studies have demonstrated an apoE isoform-specific effect on neurite outgrowth from both central nervous system- (Neuro-2a cells and GT2 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 neurites outgrowth. Our laboratory has previously shown that apoE4 inhibits and apoE3 promotes neurite outgrowth in cultured AMC neurons through the low-density lipoprotein receptor-related protein pathway (Nathan et al., 2002). It is now recognized that apoE2 and E3 gene is relatively protective against sporadic AD, while apoE4 gene increases several-fold the chances of developing the disease (Sapolsky, et al., 2000). The recent data also suggest the apparent greater risk of AD in female carriers of e4 than in males (Poirier et al., 1993; Payami et al., 1996; Rao et al., 1996). This could be attributed to an E2 deficient state combined with the e4 genotype to diminish neuronal plasticity.
Because apoE mRNA levels in the brain are induced by E2 (Srivastava et al., 1996; Stone et al., 1997), I hypothesize that E2 will support neurite outgrowth in AMC culture via increased apoE production. Consistent with this hypothesis, results from this study have suggested that E2 increases apoE secretion from AMC neurons derived from wild type mice in a dose dependent manner. Furthermore, E2 promotes neurite outgrowth from AMC neurons in a dose dependent fashion. Further support for the role of apoE in mediating E2 effects on neurite outgrowth comes from my studies on apoE KO mice. E2 has no significant effect on neurite outgrowth from AMC neurons derived from apoE KO mice, suggesting that apoE plays a vital role in mediating the effect of E2 on neurite outgrowth.

In this study by using two different model systems I have shown that E2 has isoform specific effects on neurite outgrowth in the presence of human apoE. Results from my recombinant apoE experiments clearly demonstrate that E2 could greatly promote neurite outgrowth in the presence of apoE2 or apoE3, but has no effect in the presence of apoE4, the isoform implicated in AD. These results are consistent with my studies using transgenic mice. E2 increased neurite outgrowth in AMC neurons derived from apoE3 mice, but not in apoE4 mice. How apoE isoforms modulate E2 effects on neurite outgrowth is not clear from this study.

Based on my results, I have developed a model that might help to explain the potential role of E2 and 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, and free radicals). The damage inflicted by these injurious agents signal the need for repair or
remodeling of the neurons and neurites. At this stage, the presence of E2 will maintain the secretion of apoE in the brain. If apoE2 or apoE3 is the isoform of apoE produced in the brain then they may support the repair processes. In contrast if apoE4 is produced in the brain it may not support repair very well and thus may contribute to the onset of AD.


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basis of E2's neuroprotection. Potential relevance for Alzheimer's disease. Mol 


249-302.


283-295.

receptor mRNA in the rat cerebral cortex: a nonisotopic in situ hybridization 

Apolipoprotein E immunoreactivity in cerebral amyloid deposits and 
neurofibrillary tangles in Alzheimer's disease and kuru plaque amyloid in 

Nathan, B.P., Bellosta,S., Sanan,D.A., Weisgraber, K.H., Mahley, R.W., Pitas, R.E. 
(1994). Differential effects of apolipoprotein E3 and E4 on neuronal growth in 

Nathan, B.P., Chang, K. C., Bellosta, s., Brisch, E., Ge, N., Mahley R.W., Pitas, R.E.


