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Defective Neuronal Sprouting by Human Apolipoprotein E4 Is a Gain-of-Negative Function

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The apolipoprotein E (apoE) epsilon 4 allele (apoE4) is a major risk factor for neurodegenerative conditions, including Alzheimer's disease (AD). A role for apoE in regeneration of synaptic circuitry after neural injury has been shown in several in vitro studies in which apoE3 supports neuronal sprouting better than apoE4. We evaluated sprouting in an in vitro mouse organotypic hippocampal slice culture system derived from transgenic mice expressing apoE3 or apoE4, in which apoE-dependent granule cell mossy fiber sprouting in the presence of apoE4 is only 51% of the level of apoE3. Sprouting supported by apoE4 had a dose response opposite that by supported by apoE3: although increasing E3 expression *increased* sprouting, increasing E4 expression *decreased* sprouting, suggesting that the defect in E4 in supporting neuronal sprouting is a gain-of-negative activity. These results may have important pharmacogenomic implications for AD therapies that modulate apoE expression levels.

Key words: apolipoprotein E; neurite sprouting; transgenic mice; Alzheimer's disease

Apolipoprotein E (apoE) was first described as a component of several classes of plasma lipoproteins regulating lipid metabolism. In the central nervous system (CNS), apoE is expressed by astrocytes, microglia, and, to a lesser extent, neurons (in humans). There are three human isoforms of apoE protein, E2, E3, and E4, which differ at two positions (112 and 158); E3 and E4 differ at only 112, where E3 retains a cysteine (Weisgraber, 1990). The discovery in 1993 that the apoE allele epsilon (ϵ) 4 is a major risk factor for sporadic and late-onset familial Alzheimer's disease (AD) has brought attention to the role of apoE in neurodegenerative conditions. ApoE isotype-specific activities could impact neurodegeneration and neuroregeneration through several direct and indirect mechanisms, raising the possibility that apoE isotypes differ in the pathological mechanism by which they impact AD (Ritchie and Dupuy, 1999).

ApoE is an abundant lipoprotein, which appears to be involved in membrane remodeling, repair, and lipid redistribution, especially after neuronal injury. The major epidemiological effect of the *APOE*-ϵ4 allele in AD is to accelerate the age of onset, by as much as 15 years, which may explain the three- to tenfold increased risk of AD with *APOE*-ϵ4 when age-matched comparisons are made in risk determination (Ritchie and Dupuy, 1999). In the etiology of ongoing neurodegeneration, accelerated clinical onset could be caused by defective compensatory mechanisms. Stimulating compensatory synaptogenesis, or neurite sprouting, is a major CNS activity of apoE, one in which apoE4 is clearly defective in most in vitro studies. Neurite sprouting measured in neuron cell lines and primary neurons is stimulated by apoE3, whereas E4 shows inhibitory effects, no effect, or weakly stimulatory effects on sprouting, always less than (or equal to) those of E3; the variable activity of apoE4 has been attributed, in part, to the different lipidation states of pure vs. cell-derived apoE. Results presented here extend these studies on sprouting to use of the human *APOE*-ϵ3 and *APOE*-ϵ4 transgenics (with balanced expression in all cell types) in an organotypic hippocampal slice culture (OHSC) system, in which apoE is expressed and lipidated in a physiologically relevant manner. The activity of apoE isotypes in granule neuron mossy fiber sprouting was investigated using transgenic mice that express apoE at different levels depending

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on the gene copy number. Because apoE expression is up-regulated in glial reactive responses to neurodegeneration, the dose responses of apoE activities could play an important role in its activity in AD. Results show that apoE3 and apoE4 are qualitatively different in their abilities to support neuronal sprouting when apoE expression levels increase.

MATERIALS AND METHODS

Animals

C56Bl/6J (Jackson Laboratories, Bar Harbor, ME) and C56Bl/6J-ApoE<tm1Unc> [apoE-ko (Piedrahita et al., 1992) tenth-generation back-cross to C56Bl/6J by Jackson Laboratories] mice were maintained as inbred colonies. Human *APOE*-ε3 and *APOE*-ε4 (driven by the human *APOE* promoter) transgenic mice were made in the apoE-ko background and show equivalent levels of expression in CNS cells (Xu et al., 1996). Human apoE isoforms are expressed in astrocytes, microglia, and, to a lesser extent, neurons, as seen in humans [which is determined by the human *APOE* promoter elements (Roses et al., 1998)], and maintains, to an unknown extent, the human pattern of expression regulation and regional expression (Xu et al., 1996, 1998, 1999). Hemizygous (one gene copy) and homozygous (two gene copies) animals were generated by hemizygous crosses. Both littermates and nonlittermates were compared in all analyses; no significant effects between litters were detected in any analyses (data not shown). *APOE*-ε3 and *APOE*-ε4 transgene copy number was determined by quantitative PCR amplification of tail-cut DNA samples; mouse apoE gene copy number was determined by using genotyping primers that detect both wild-type and knockout genes (data not shown). All surgical and animal care procedures were carried out in strict adherence to NIH Publication No. 80-23. Mice were kept on a 12 hr light-dark cycle and provided food and water ad libitum.

OHSC

Postnatal day 7 pups were anesthetized with CO_2 and hippocampal slice cultures prepared as described elsewhere (Stoppini et al., 1991), with some modifications (Harris-White et al., 1998). Hippocampi were sliced to 400 μm, placed on a membrane insert (Costar, Cambridge, MA) with 1.2 ml media consisting of 64% minimal essential medium + HEPES (Gibco, Grand Island, NY), 32% Hank's balanced salt solution (Sigma, St. Louis, MO), 6.5 mg/ml glucose, penicillin-streptomycin (50 U/ml; 0.05 mg/ml), and the serum substitute TCM (final concentration 2%; ICN Biomedicals, Costa Mesa, CA). The medium was changed every 2 days for the 6 days in vitro (DIV), then every 3 days. Cultures were maintained for 18 DIV.

Timm's Heavy Metal Staining

The Timm's stain procedure was adapted for slice cultures as described elsewhere (Zimmer and Gahwiler, 1987). Briefly, at 18 DIV, cultures are treated with 1% Na_2S for 10 min and developed with buffered $AgNO_3$ for 35–40 min at 26°C. The membrane was mounted on slides in Permount for image analysis.

Quantitation of Mossy Fiber Sprouting

Timm's stained mossy fiber sprouting was quantitated as previously described (Coltman et al., 1995), with minor modifications (Teter et al., 1999a). Briefly, digitized images of slices were video captured using NIH Image software, with treatment group blind to the operator, and the Timm's staining was quantitated in two supragranular regions, ventral and dorsal. Optical density was measured in a 100 × 50 μm box placed over the three most intensely staining areas within each of the dorsal, ventral, and central hilar regions. The two supragranular measures were corrected by subtracting background transmittance measurements taken in the adjacent hippocampal fissure, then dividing by the hilus measurement, which gives the sprouting index; this normalization to the hilus eliminates variability because of Timm's staining differences between cultures and between experiments (Coltman et al., 1995). The sprouting indices were analyzed by ANOVA with Fisher's PLD post hoc test to assess the significance of differences between groups and by testing the equality of slopes for differences between regression analyses (Sokal and Rohlf, 1981).

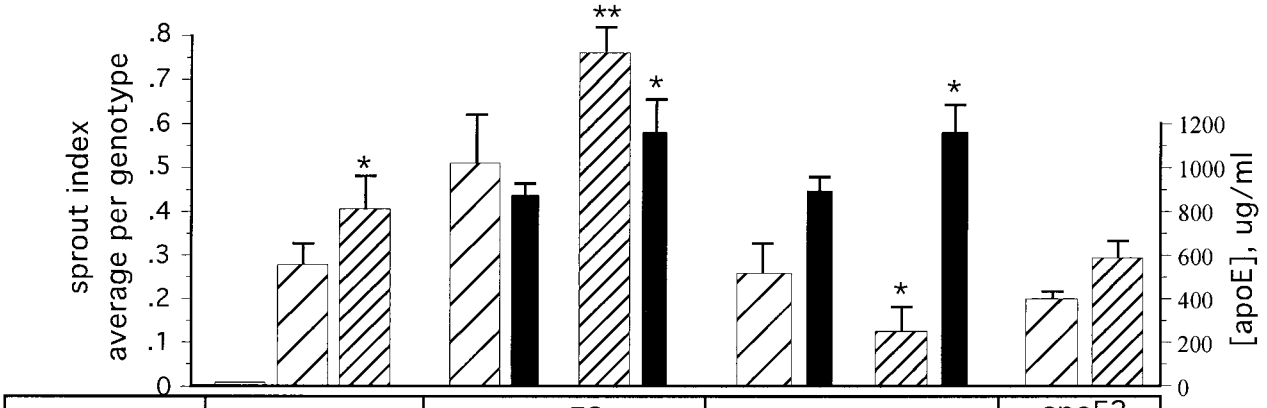
Human ApoE ELISA

Media from OHSC were collected at DIV 18, after 3 days of conditioning. Human apoE was measured by sandwich ELISA, using 2E1 monoclonal antibody to apoE (Boehringer-Mannheim, Indianapolis, IN) to capture and goat anti-human apoE antibody (Chemicon, Temecula, CA) to detect, with alkaline phosphatase as reporter, as described elsewhere (Gracia et al., 1994).

RESULTS

Mossy fiber sprouting in OHSC was previously shown to be dependent on mouse apoE expression (Teter et al., 1999a) and on human apoE isotype (Teter et al., 1999b). Because apoE4 is defective compared with apoE3, we evaluated the nature of the apoE4 defect by measuring mossy fiber sprouting in OHSC as a function of human apoE isotype expression level, when modulated by apoE gene copy number; hemizygous (one transgene copy) and homozygous (two transgene copies) animals were generated by hemizygous crosses. Mossy fiber sprouting was measured by image analysis of Timm's stained mossy fibers that sprout into the dorsal (suprapyramidal) outer molecular layer of the dentate gyrus, expressed as a sprouting index (see Materials and Methods).

For one-gene animals, apoE4 showed significantly less sprouting than apoE3 (Fig. 1); the sprouting index for apoE4 was only 51% of that of apoE3 ($P < 0.01$), essentially as previously observed (Teter et al., 1999b). For both apoE3 and apoE4, one-gene animal sprouting indices were significantly different from those of two-gene animals. However, the direction of change was opposite: whereas two-gene apoE3 showed a 49% increase in the sprouting index (0.51 ± 0.11 vs. 0.76 ± 0.06; $P < 0.01$), two-gene apoE4 showed a 54% decrease (0.26 ± 0.06 vs. 0.12 ± 0.05; $P < 0.01$). To rule out the possibility that the lower initial sprouting levels of apoE4 precluded the ability to increase sprouting when the gene dose is doubled, apoE3 cultures were evaluated at a time point when the



apoE gene	mouse apoE			apoE3		apoE4		apoE3 (14 DIV)	
gene copy #	0	1	2	1	2	1	2	1	2
# OHSC	28	55	35	95	34	70	50	24	13
# animals	2	3	2	8	3	7	4	2	1

Fig. 1. Mossy fiber sprouting index and apoE protein levels in OHSC. The mean sprouting indices (hatched bars) for one-gene and two-gene mouse *APOE* and human *APOE*-ϵ3 (apoE3) and *APOE*-ϵ4 (apoE4) transgenic mice OHSC are shown as well as human apoE protein levels as determined by ELISA (black bars). Cultures were terminated at 18 DIV, except where indicated. Note that the sprouting index of apoE3 (one-gene) at 14 DIV was equivalent to that of apoE4 (one-gene) at 18 DIV and that the apoE3 two-gene cultures showed a trend of increased sprouting even at this earlier point. Differences between one-gene and two-gene groups were statistically significant, as indicated (*$P < 0.05$, **$P < 0.01$); standard deviations are shown. The number of OHSC slices and animals from which they were derived are indicated; for culture, two wells were used for each animal containing OHSC from one hippocampus each.

sprouting index was similar to that of apoE4. ApoE3 cultures were prematurely terminated (at 14 DIV) to make the sprouting index of one-gene apoE3 equivalent to that of one-gene apoE4 cultures at 18 DIV. The increased sprouting in two-gene apoE3 cultures was still seen even at this lower one-gene sprouting level (Fig. 1).

Mouse apoE is similar, in part, to human apoE4 in the critical cysteine/arginine residues at 112 and 158, yet it has many other differences that could modify an apoE4-like phenotype [notably, the absence of arginine at 61, which is critical for apoE4-specific activity (Weisgraber, 2001)]. To determine whether mouse apoE had activity like that of apoE4, the phenotype of mouse apoE was tested by similar copy number manipulation, using hemizygous crosses to generate mice with zero, one, or two mouse apoE gene copies. As shown in Figure 1, the complete lack of sprouting in zero-gene OHSC (essentially apoE-knockout) reflects the apoE dependence of this sprouting paradigm, as previously described (Teter et al., 1999a). Mouse apoE showed a 52% increase in the sprouting index between one- and two-gene OHSC (0.27 ± 0.05 vs. 0.41 ± 0.08; $P < 0.05$; Fig. 1). These results indicate that mouse apoE behaves qualitatively similarly to human apoE3 in this sprouting activity.

To modulate apoE expression levels and measure the effect on sprouting, human apoE transgene copy number was varied. The OHSC derived from one-gene and two-gene animals showed proportional levels of apoE protein in the culture media (as measured by ELISA), as shown in Figure 1; variable levels of apoE in different wells (Fig. 2) were largely caused by different numbers of surviving slices in each well (this varied from five to nine slices per well and was proportional to the apoE level in each well; data not shown). The average sprouting index of the five to nine slices in each well was plotted against the level of apoE protein in the well medium (Fig. 2). For apoE3, sprouting was positively correlated with increased apoE levels, whereas, for apoE4, sprouting was negatively correlated with increased apoE levels; linear regression analysis showed slopes of $+7 \times 10^{-4}$ for apoE3 and -5×10^{-4} for apoE4 and R^2 values of 0.61 for apoE3 and 0.59 for apoE4. Irrespective of gene copy number, variations in apoE protein levels were strong determinants of the sprouting index. These results show that these two apoE isotypes are qualitatively different in their dose-dependent support of mossy fiber sprouting. ApoE3 showed increased sprouting with increasing expression, indicative of a positive activity for sprouting, whereas apoE4 showed decreased sprouting with increasing expression, indicative of a gain-of-negative activity.

DISCUSSION

ApoE4 influences the risk of AD through its pleiotropic effects on both the pathology of AD and the environmental and developmental factors influencing its etiologies (Teter, 2000; Teter et al., 2002). Clearly, apoE4 influences neurodegeneration in AD (Poirier, 1994) and age-dependent neurodegeneration in apoE4 transgenic mice (Buttini et al., 1999). This may be related to its effects on Ca^{2+}-dependent neurotoxicity (Marques et al., 1996;

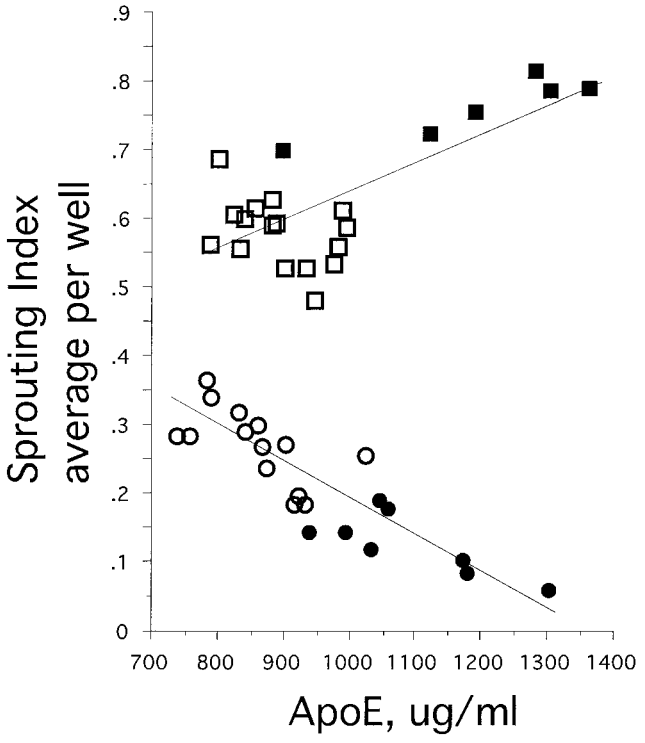


Fig. 2. The relationship between the sprouting index and the apoE protein level is isotype dependent. The OHSC derived from one-gene and two-gene animals (open and solid symbols, respectively) expressed variable levels of apoE protein in the well culture media (see Results), and this is plotted against the average index of mossy fiber sprouting for all slices in the well (for clarity, error bars of individual well sprouting indices are omitted; the average sprouting index standard deviation was ± 0.08). Linear regression analysis showed that apoE3 (squares) had a slope of $+7 \times 10^{-4}$, whereas apoE4 (circles) had a slope of -5×10^{-4}. The R^2 values were 0.61 for E3 and 0.59 for E4. A test utilizing all individual slice sprouting indices (not the mean values shown here) indicated that the slopes of the regression analyses are different ($P < 0.0001$).

Muller et al., 1998), cholesterol metabolism (Michikawa and Yanagisawa, 1998), or oxidative damage (Miyata and Smith, 1996; Ramassamy et al., 1999; Pedersen et al., 2000; Jolivalt et al., 2000). The regenerative capacity of the CNS can, in principle, counteract some neurodegeneration, yet behavioral deficits are persistent in apoE4 transgenics (Raber et al., 1998, 2000; Buttini et al., 1999). Regeneration of synaptic circuitry in response to neurodegeneration could be one mechanism that delays the onset of AD, explaining an important epidemiologic defect in apoE4 populations (for reviews see Mesulam, 1999; Arendt, 2001; Teter, 2000b).

The mechanism by which apoE facilitates neuronal sprouting may involve lipid trafficking. Recently, apoE and the cholesterol it carries were identified as the glial factor that stimulates new synapse formation in cultured neurons (Mauch et al., 2001). The activity of apoE in supporting neuronal regeneration has received much support, with apoE4 consistently showing defects. In most studies, apoE4 was defective in supporting neurite sprouting (Table I). Possible mechanisms include isotype-specific effects on lipid efflux (Michikawa et al., 2000), apoE cellular accumulation (Nathan et al., 1995; Ji et al., 1998), microtubule metabolism (Nathan et al., 1995), or neurotoxicity (Marques et al., 1996) (for reviews see Teter, 2000, 2002; Teter et al., 2002). Critical issues yet to be fully resolved are the effects of the lipidation state of apoE and neuronal expression of human apoE. Results presented here use human *APOE*-ε3 and *APOE*-ε4 transgenics that express apoE in vivo with cellular specificity like that seen in humans (Xu et al., 1996, 1998, 1999) and, therefore, in a physiologically relevant lipidation state. In the OHSC system, not only is apoE4 partially defective in supporting mossy fiber sprouting compared with apoE3 but increased levels of apoE4 further exacerbated the defect, indicating that the defect in apoE4 is a gain-of-negative activity. Studies using dorsal root ganglion and primary cortical neurons have shown a similar dose-dependent effect with similar concentrations of pure E3 and E4 (Nathan et al., 1994, 2002), although E4 did not stimulate the basal (apoE-independent) sprouting; in contrast, in the OHSC system, basal sprouting (that of apoE-knockout mice) is essentially zero (Fig. 1), and apoE4 showed weak sprouting activity. The apparent gain-of-negative activity of apoE4 could be a form of toxicity that, at higher doses, dominates its weak sprouting activity. This could be relevant at the apoE levels measured in OHSC media, because similar levels are found in human cerebrospinal fluid and brain (2–6 μg/ml; Hesse et al., 2000).

Because apoE expression is increased in most neurodegenerative conditions as part of glial activation responses, the dose responsiveness of isotype-specific activities bears not only on our understanding of isotype-specific effects but also on the therapeutic implications of altering apoE expression levels. However, this has received little experimental attention. In addition to the present work, another recent application of the transgenic approach has revealed a similar gene-dose response to neurodegeneration, in which two-copy apoE4 animals had greater loss of synaptic markers (synaptophysin, MAP2, and neurofilament) at 8 months (Buttini et al., 2000). In addition, apoE4 was dominant over apoE3 in these measures, similar to results for sprouting in vitro (Holtzman et al., 1995; Nathan et al., 1995). These results may provide insight into the mechanism of apoE4 in AD, in which apoE4 reduced dendritic remodeling of pyramidal and subcortical neurons; *APOE*-ε4 copy number affected the relationship and possible "coupling" between neuronal degeneration and dendritic growth, with ε4/ε4 showing no relationship and a shift toward proximal branching (for review see Arendt, 2001). The latter effect of apoE4 on branching is seen in vitro (Nathan et al., 1995) and may explain the reduced distal mossy fiber sprouting measured here in OHSC (outer molecular layer sprouting).

The gain-of-negative function of E4 could have important clinical implications for the pharmacogenomic

TABLE I. ApoE4 Is Defective in Supporting Neurite Sprouting In Vitro

ApoE source	Neurite source	ApoE4 effect (apoE3 stimulates)	Depends on	References
Pure	DRG and 1° cortical neuron	Inhibits	Lipoprotein, apoE levels	Handelmann et al., 1992; Nathan et al., 1994; Nathan et al., 2002
Pure	N2A	Inhibits	β-VLDL, LDLR/LRP	Nathan et al., 1994; Nathan et al., 1995
Transfected N2A low expressing	N2A	Inhibits	β-VLDL, HSPG/LRP	Bellosta et al., 1995
Transfected N2A high expressing	N2A	Neutral		DeMattos et al., 1998
Pure	GT1-1 (a HT line)	Neutral	β-VLDL, LRP	Holtzman et al., 1995
Human plasma HDL, CSF lipoproteins	GT1-1 (a HT line)	Neutral	LRP	Fagan et al., 1996
GFAP transgenic astrocyte	1° HC neuron	Neutral	LRP	Sun et al., 1998
Pure (no lipid) + laminin	1° HC neuron	Stimulates (=E3)		Huang et al., 1995
Transfected HEK cells	1° HC neuron	Stimulates (=E3)		Puttfarcken et al., 1997
Human *APOE* transgenic OHSC	Granule neurons	a) Stimulates (=58% of E3) b) "Inhibits" by dose	b) ApoE levels	a) Teter et al., 1999b b) This study

efficacy of therapeutic drugs that impact or target apoE expression (Poirier, 1999; Saunders et al., 2000). For example, the efficacy of estrogen replacement therapy (ERT) in improving the cognitive deficits in postmenopausal women with AD (van Duijn et al., 1996) may reflect stimulation of neurite sprouting (Stone et al., 1998; Teter et al., 1999a) through stimulation of apoE expression (Stone et al., 1997). Indeed, *APOE*-ε4 women show no benefit of ERT (Yaffe et al., 2000) and showed less response to tacrine (anticholinesterase) therapy (Schneider and Farlow, 1997). It will be important to evaluate apoE genotype effects in trials of other drugs that can modulate apoE expression.

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