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Human Apolipoprotein E Isoform-Specific Differences in Neuronal Sprouting in Organotypic Hippocampal Culture

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Abstract: The apolipoprotein E (ApoE) ϵ4 allele is a major risk factor for neurodegenerative conditions, including Alzheimer's disease. A role for ApoE is implicated in regeneration of synaptic circuitry after neural injury. In the in vitro mouse organotypic hippocampal slice culture system, we previously showed that cultures derived from ApoE-knockout mice are defective in mossy fiber sprouting into the dentate gyrus molecular layer. This sprouting defect was rescued in cultures from transgenic mice expressing ApoE3 under the control of the human promoter and in ApoE-knockout cultures treated with ApoE3-conditioned media. Although the ApoE3 transgene fully restored sprouting, ApoE4 restored sprouting to only 58% of ApoE3 levels. These data indicate that ApoE isoform-specific effects on neuroregeneration may contribute to its genetic risk for Alzheimer's disease. **Key Words:** Apolipoprotein E—Neurite sprouting—Transgenic mice—Alzheimer's disease.
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The adult CNS and PNS respond to injury with limited yet effective regeneration of synaptic circuitry. Accumulating evidence implicates a role for apolipoprotein E (ApoE) in the mechanism of recovery (Poirier, 1994; Weisgraber et al., 1994; Laskowitz et al., 1998). ApoE has many activities that could account for its effects on CNS and PNS plasticity after injury, with cholesterol and phospholipid metabolism consistently implicated (Mahley, 1988). One model for this role uses glia recycling cholesterol from scavenged neuronal membrane, repackaging it with ApoE for neuronal uptake and neurite growth (Poirier, 1994; Laskowitz et al., 1998).

ApoE genotype is a major risk factor for the outcome of acute neurodegeneration (Laskowitz et al., 1998) and for Alzheimer's disease (AD), where the ϵ4 allele accelerates age of onset (Corder et al., 1993). In AD there is extensive loss of entorhinal cortex (EC) neurons (Hyman et al., 1986) and loss of synaptophysin immunoreactivity in its major projection, the hippocampus (Heinonen et al., 1995). Regenerative sprouting in the hippocampus (Geddes et al., 1985; Arendt et al., 1997) may reflect functions of ApoE in compensatory events.

Mechanisms of axonal sprouting in response to neural deafferentation have been investigated in the in vitro mouse organotypic hippocampal slice culture (OHSC) system. This system develops and retains organotypic features and events of the intact hippocampus (Stoppini et al., 1993; Gahwiler, 1998), including development of the mossy fiber pathway that arises from dentate granule cells and projects to the CA3 pyramidal cells (Zimmer and Gahwiler, 1987), as well as other synaptic development phenomena that parallel those observed in vivo (Gahwiler, 1998). The preparation of hippocampal slices removes the major extrinsic innervation by the EC to the granule cell dendritic field in the outer molecular layer and the commissural projection to the inner molecular layer (Frotscher, 1992). This deafferentation stimulates sprouting of granule cell mossy axon collaterals into the molecular layer, where they are not normally found in abundance (Zimmer and Gahwiler, 1987; Sekiguchi et al., 1996). There they make aberrant synapses with dendrites of the deafferentated granule cells that are electrophysiologically functional (Stoppini et al., 1993). We previously showed that sprouting is regionally defective in ApoE-knockout (ApoE-ko) OHSC and that estrogen stimulates the normal sprouting response in the same region (Teter et al., 1999). In the present study, we tested the relative activities of ApoE3 and ApoE4 in rescuing this ApoE-dependent sprouting.

EXPERIMENTAL PROCEDURES

Animals

C56Bl/6J (Jackson Labs) and C56Bl/6J-ApoEtm1Unc (ApoE-ko; 10th generation backcross to C56Bl/6J by Jackson Labs) mice were maintained as inbred colonies. Human ApoE3 and ApoE4 (driven by the human ApoE promoter) transgenic mice were made in the ApoE-ko background (Xu et al., 1996). All surgical and animal care procedures were carried out with strict adherence to the NIH *Guide for the Care and Use of Laboratory Animals* (publication no. 80-23). Mice were kept on a 12-h light–dark cycle and provided food and water ad libitum.

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Abbreviations used: AD, Alzheimer's disease; ApoE, apolipoprotein E; ApoE-ko, apolipoprotein E-knockout; DIV, days in vitro; EC, entorhinal cortex; OHSC, organotypic hippocampal slice culture.

OHSC preparations

Postnatal day 7 pups were anesthetized with CO_2, and hippocampal slice cultures were prepared as described (Stoppini et al., 1991) with some modifications (Harris-White et al., 1998). Hippocampi were sliced to 400 μm and placed on a membrane insert (Costar) with 1.2 ml of medium consisting of 64% minimal essential medium plus HEPES (GIBCO), 32% Hanks' balanced salt solution (Sigma), 6.5 mg/ml glucose, penicillin/streptomycin (50 U/ml and 0.05 mg/ml), and the serum substitute TCM (final concentration, 2%; ICN Biomedicals). The medium was changed every 2 days for 6 days in vitro (DIV) and 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 (Zimmer and Gahwiler, 1987). In brief, at 18 DIV, cultures were 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.

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., 1999). In brief, digitized images of slices were video-captured using NIH Image software, with the operator blinded to the treatment group, and the Timm's staining was quantitated in two supragranular regions, ventral and dorsal. Optical density was measured in a 100- $\times$ 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 and then dividing by the hilus measurement, which gives the sprouting index; this normalization to the hilus eliminates variability due to 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.

Human ApoE ELISA

Human ApoE was measured by sandwich ELISA, using 2E1 monoclonal antibody to ApoE (Boehringer-Mannheim) to capture and goat anti-human ApoE antibody (Calbiochem) to detect, with alkaline phosphatase as reporter, as described (Gracia et al., 1994).

RESULTS

Mossy fiber sprouting in wild-type and ApoE-ko mouse OHSCs

Wild-type C57B16/J OHSCs show typical sprouting fibers that originate in the hilar region and project through the granule cell layer to the molecular layer in the dorsal and ventral regions (Fig. 1a and b). In contrast, cultures derived from ApoE-ko mice did not show Timm's stained sprouting of mossy fibers from the dorsal region of the hilus (Fig. 1c), although ventral region sprouting (V in Fig. 1b) was not different from that for C57B16/J cultures and was therefore ApoE-independent (Teter et al., 1999).

Rescue of dorsal region sprouting by human ApoE isoforms

The ability of human ApoE isoforms to rescue the dorsal region ApoE-dependent sprouting was tested using cultures from transgenic mice expressing ApoE3 or ApoE4 under the control of the human promoter. Both ApoE3 (Fig. 1d, arrows) and ApoE4 isoforms showed sprouting in the dorsal region (Table 1), indicating they rescued the sprouting defect seen in their background strain, ApoE-ko. Quantitative analysis of Timm's staining showed that ApoE3 fully restored dorsal region sprouting (Table 1). This level of sprouting was higher than that of C57B16/J mice, although quantitative biological conclusions about the relative activities of mouse ApoE versus human ApoE3 are precluded because their relative expression levels could not be precisely measured. ApoE4 also rescued sprouting but to a level only 58% of ApoE3 levels (Table 1). Ventral region sprouting did not differ between ApoE3 and ApoE4 (data not shown), consistent with its ApoE independence (Teter et al., 1999). ApoE3 and ApoE4 culture media contained equivalent levels of ApoE, which did not vary among media collected at 4, 9, and 15 DIV. ApoE4 cultures contained 924 $\pm$ 62 ng/ml ApoE (n = 4 wells), and ApoE3 cultures contained 839 $\pm$ 86 ng/ml (n = 7 wells), in medium conditioned for 2 days, as measured by ELISA. The ApoE3 and E4 transgenes are expressed in the brain at levels comparable to mouse ApoE in C57B16/J mice (Xu et al., 1996).

Rescue of sprouting by ApoE3-conditioned medium

The ability of medium conditioned by ApoE3 OHSCs to rescue the defective sprouting was tested by treating ApoE-ko OHSCs. Medium was conditioned by ApoE3 OHSCs for 2 days, from 10 to 12 DIV, collected, filter-sterilized and frozen at −80°C. Conditioned medium was applied to the ApoE-ko OHSCs from 4 to 18 DIV (100% conditioned medium) and changed every 2 days. The level of ApoE3 in the conditioned medium was 725 ng/ml before treatment and 531 $\pm$ 148 ng/ml after 2 days of treatment, as measured by ELISA. Dorsal region mossy fiber sprouting was partially rescued to 20% that of the ApoE3 transgenic sprouting (Table 1).

DISCUSSION

Although ApoE deficiency does not compromise PNS regeneration (Popko et al., 1993), it apparently does so in the CNS (Masliah et al., 1995; Anderson et al., 1998). The CNS defects of ApoE-ko mice include synaptic neurochemical derangements that parallel AD (Chapman and Michaelson, 1998), exacerbated injury responses (Laskowitz et al., 1998), impaired memory and learning, which can be corrected by ventricular infusion of human ApoE (Masliah et al., 1997), and age-dependent neurodegeneration, which can be corrected by human ApoE expression (Buttini et al., 1999). However, some ApoE-ko phenotypes are not consistently observed (Anderson et al., 1998). Our previous studies in ApoE-ko cultures showed that ApoE is required for sprouting of mossy fibers in the dorsal dentate gyrus (of the hippocampus) (Teter et al., 1999), consistent with the impaired synaptic regeneration in ApoE-ko mice in response to EC deafferentation in vivo (Masliah et al., 1995; Anderson et al., 1998). The defect in ApoE-ko sprouting was partially rescued by medium conditioned by ApoE3 cultures, indicating that granule cells in the dorsal region of ApoE-ko cultures are competent for sprouting and that the stimulatory effect is mediated, at least in part, by a secreted molecule (most likely ApoE); however, it is not known whether the limited rescue (20%) resulted from limitations in the ApoE-ko tissue or the conditioned medium.

ApoE3 rescued the dorsal region sprouting that was defective in ApoE-ko cultures. ApoE4 was only 58% as effective as ApoE3 in rescuing sprouting in this transgene expression-matched system

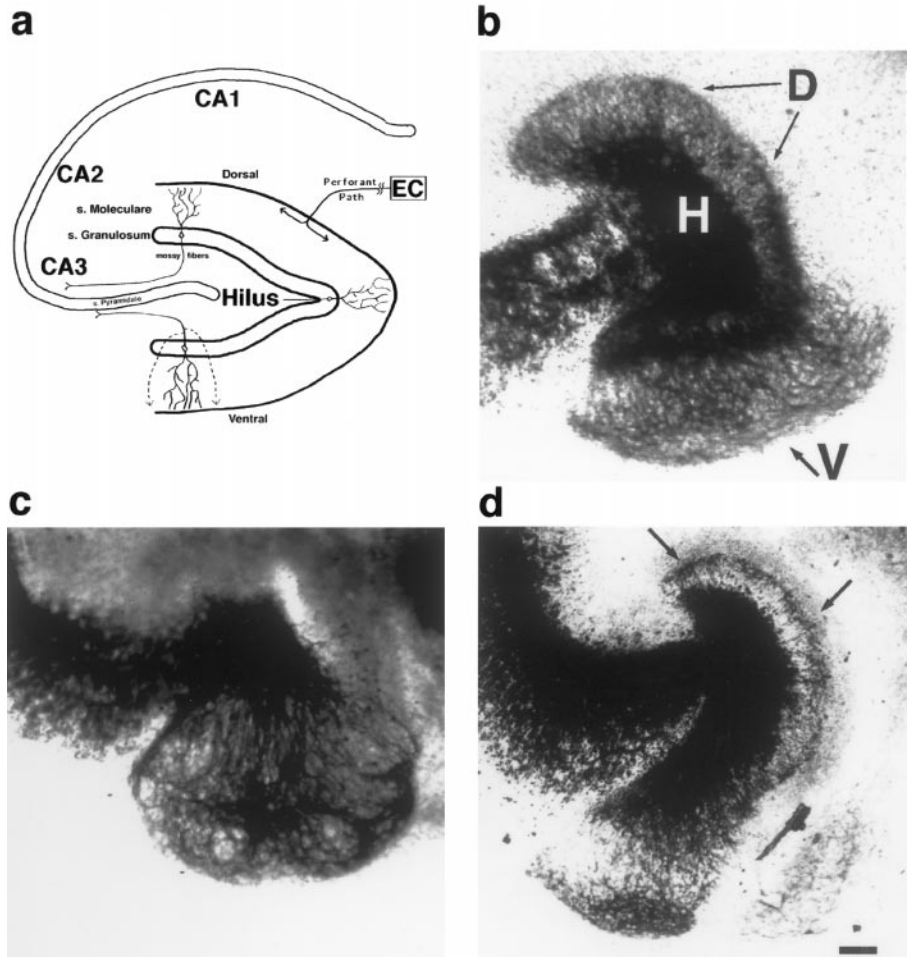

FIG. 1. Mossy fiber sprouting in hippocampal slice cultures. **a:** Diagram of hippocampus shows granule cell dendritic tree in the molecular layer with its innervation from the perforant path from the EC, the mossy fibers from the hilus projecting through CA3 to the left, and representations of sprouting mossy fibers indicated by dashed lines in the ventral region. Also shown are Timm's-stained OHSCs after 18 DIV (with CA3 projecting to the left) derived from C57B1/6J (**b**), ApoE-ko (**c**), and human ApoE3 transgenic (**d**) mice. H, hilus; D, dorsal sprouting region; V, ventral sprouting region. Bar = 0.1 mm.

(E3 expression = E4). These results in the OHSC system, which retains in vivo anatomical and cellular interactions and produces native apolipoprotein particles with ApoE expression regulated by the human promoter, validate previous observations of an ApoE4 defect compared with ApoE3 in stimulating neurite sprouting in purified neuron cultures (Bellosta et al., 1995; Holtzman et al., 1995; DeMattos et al., 1998; Sun et al., 1998). However, these results from OHSCs do not distinguish between the defective neuroregenerative effects of ApoE4 and neurodegenerative effects that are exacerbated by ApoE4 (Bellosta et al., 1995; Sheng et al., 1998).

Whether compensatory growth is widespread and whether it reverses cognitive deficits is not clear (Cotman et al., 1991; Masliah et al., 1991; Poirier, 1994). Regenerative sprouting in the dentate gyrus (of the hippocampus) was shown in a subset of AD patients with unknown ApoE genotype (Geddes et al., 1985) and was suggested by increased Timm's staining in aged human (Cassell and Brown, 1984). However, AD patients with ApoE4 show reduced dendritic remodeling of pyramidal and subcortical neurons (Arendt et al., 1997). These results indicate that human isoform-specific effects of ApoE on neuroregeneration may be relevant to its mechanisms of risk for AD and synergism with prior head injury (O'Meara et al., 1997) as well as its risk for ischemic injury and outcome of stroke (Sheng et al., 1998). If so, correction of the ApoE4 sprouting defect may have a clinical impact.

TABLE 1. *Quantitative analysis of Timm's-stained mossy fiber dorsal sprouting*

	C57B16/J	ApoE-ko	ApoE-ko + E3	ApoE3	ApoE4
Sprouting index	0.39	<0.01	0.13	0.51	0.30[a]
SD	0.04	ND	0.08	0.06	0.03
No. of slices	43	47	44	61	81
No. of animals	3	4	4	5	7
Interanimal SD	0.03	ND	0.04	0.05	0.03

The sprouting index and statistical parameters are given for cultures from four strains of mice: C57B16/J (control), ApoE-ko (background for ApoE3 and ApoE4 transgenics; see Experimental Procedures), ApoE3, and ApoE4. ApoE-ko cultures were also treated with medium conditioned by ApoE3 cultures. ND, not detectable.

[a] $p < 0.001$ compared with ApoE3.

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