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ROLE OF APOLIPOPROTEIN E AND ESTROGEN IN MOSSY FIBER SPROUTING IN HIPPOCAMPAL SLICE CULTURES

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Abstract—A role for apolipoprotein E is implicated in regeneration of synaptic circuitry after neural injury. The *in vitro* mouse organotypic hippocampal slice culture system shows Timm's stained mossy fiber sprouting into the dentate gyrus molecular layer in response to deafferentation of the entorhinal cortex. We show that cultures derived from apolipoprotein E knockout mice are defective in this sprouting response; specifically, they show no sprouting in the dorsal region of the dentate gyrus, yet retain sprouting in the ventral region. Dorsal but not ventral sprouting in cultures from C57Bl/6J mice is increased 75% by treatment with 100 pM 17β-estradiol; this response is blocked by both progesterone and tamoxifen.

These results show that neuronal sprouting is increased by estrogen in the same region where sprouting is dependent on apolipoprotein E. Sprouting may be stimulated by estrogen through its up-regulation of apolipoprotein E expression leading to increased recycling of membrane lipids for use by sprouting neurons. Estrogen and apolipoprotein E may therefore interact in their modulation of both Alzheimer's disease risk and recovery from CNS injury.

Key words: apolipoprotein E, estrogen, neurite sprouting, Alzheimer's disease.

The adult central and peripheral nervous systems (CNS and PNS) respond to injury with limited yet effective regeneration of synaptic circuitry which re-establishes lost function. Although little is known about the mechanism of recovery, accumulating evidence implicates a role for apolipoprotein E (ApoE), possibly through its function in membrane lipid recycling.[52,53] ApoE expression increases in reactive responses of both astrocytes and microglia which, in turn, play critical roles in CNS response to and recovery from injury.[10] ApoE in the brain represents an endogenous pool synthesized by glia. Although this has been attributed primarily to astrocytes,[51,78] we and others have shown that microglia express ApoE.[47,71,75]

ApoE genotype is a major risk factor for Alzheimer's disease (AD) where isotype E4 increases risk and accelerates age of onset (reviewed in Roses and Saunders[58]). In AD there is extensive loss of entorhinal cortex (EC) neurons,[28] loss of synaptophysin immunoreactivity in the hippocampus,[26,60] and neuronal cytoskeletal disruption.[42] A subset of patients with unknown ApoE genotype have shown regenerative sprouting in the dentate gyrus (DG).[23]

Studies of the ApoE knockout (ApoE-KO) mouse[50] have revealed insight into ApoE's functions, both peripherally and centrally. ApoE knockout mice, although neuropathologically normal, show numerous CNS defects including impaired memory and learning deficits, some of which are age-dependent.[24,31,40,41,77] They also show cholinergic changes[24] and age-related disruption in the dendritic cytoskeleton and reduced synaptophysin and microtubule-associated protein-2 immunoreactivity in the hippocampus[37] (reviewed in Ref. 40). ApoE-KO mice also show defects in responses to injury, including cerebral ischemia,[13,32] and impaired synaptic regeneration (recovery of synaptophysin-IR) in the outer molecular layer (OML) after EC lesion (ECL).[38] However, some of these effects are not consistently observed.[1a,22,40] In addition to manipulating ApoE by gene knockout, functional effects of elevating ApoE levels in brain and CNS cells *in vitro* have been shown by estrogen[68,71] and by ventricular infusion of human ApoE into the ApoE-KO mouse correcting the cognitive and neuron structure defects.[41]

The hippocampus is a model system for synaptic plasticity and re-organization after injury because of its roles in learning and memory as well as its status as a locus for many acute and degenerative conditions.[61] Mechanisms of axonal sprouting in response

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Abbreviations: AD, Alzheimer's disease; ApoE, apolipoprotein E (gene and protein); ApoE-KO, (also, EKO) ApoE "knockout" mouse, targeted-mutation transgenic eliminating ApoE expression; BSA, bovine serum albumin; C57, C57Bl/6J mouse strain; DG, dentate gyrus (of the hippocampus); DIV, days *in vitro*; EC, entorhinal cortex; ECL, EC lesion; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; IML, inner molecular layer (of the dentate gyrus); IR, immunoreactivity; LDLR, low density lipoprotein; ML, molecular layer; OHSC, organotypic hippocampal slice culture; OML, outer molecular layer (of the dentate gyrus); PD, postnatal day; PNS, peripheral nervous system; RT, room temperature; TBS, Tris-buffered saline.

to neural deafferentation have been investigated in an *in vitro* mouse organotypic hippocampal slice culture (OHSC) system. This system mimics many *in vivo* events and therefore provides a powerful system to identify relevant mechanisms. OHSC continues to develop and retains organotypic features of the intact hippocampus,[2,6,72] including development of the mossy fiber pathway that arises from dentate granule cells and projects to the CA3 pyramidal cells,[74,81] as well as other synaptic development phenomena that parallel those observed *in vivo*.[21,45]

The preparation of hippocampal slices transects the perforant path, thereby removing the major extrinsic innervation by the EC to the granule cell dendritic field in the OML, as well as the commissural projection to the inner molecular layer (IML).[19] This deafferentation stimulates sprouting of granule cell mossy axon collaterals into the molecular layer (ML), where they are not normally found in abundance.[20,63,81] There they make aberrant synapses with dendrites of the deafferented granule cells ("reactive synaptogenesis") that are electrophysiologically functional.[21,72]

EXPERIMENTAL PROCEDURES

Animals

C56Bl/6J and C56Bl/6J-ApoEtm1Unc (ApoE-knockout) mice were obtained from Jackson Labs and maintained as an inbred colony. The ApoE-knockout mice were the 10th generation backcross to C56Bl/6J by Jackson Labs. To minimize animal suffering, all surgical and animal care procedures were carried out with strict adherence to the guidlines set out in the NIH guide for the care and use of laboratory animal, NIH Publication no. 80–23. Mice were kept on a 12 h light–dark cycle and provided food and water *ad libitum*.

Organotypic hippocampal slice culture

Postnatal day (PD) 7 pups were anesthetized with CO_2, sterilized and decapitated. Hippocampal slice cultures were prepared according to the method of Stoppini *et al.*[73] with some modifications.[25] Hippocampi from PD7 pups were sliced to 400 μm on a Stoelting tissue chopper and arranged in anatomical order (septal to temporal) in a 24 mm-diameter 0.4 μm-pore clear membrane insert (Costar) in a six-well tissue culture tray containing 1.2 ml media. The medium was changed every two days for the five days *in vitro* (DIV), then every three days. Slice culture medium consisted of 64% minimal essential medium + HEPES without Phenol Red (Gibco), 32% Hanks' balanced salt solution without Phenol Red (Sigma), 6.5 mg/ml glucose, penicillin-streptomycin (50 U/ml–0.05 mg/ml), and the serum substitute TCM™ (final concentration 2%; ICN Biomedicals) which provides 675 μg/ml total protein (final concentration) consisting of a proprietary mixture of bovine serum albumin (BSA)(heat-treated and fatty acid-free), transferrin, and insulin (the latter two have been shown to be necessary and sufficient for neuron growth and survival *in vitro*[1]).

The degree of mossy fiber sprouting is dependent on the position of the slice in the septal-temporal axis of the hippocampus; sprouting is greatest in septal slices, is slightly reduced in medial slices, and is greatly reduced in temporal slices.[8,12] For this reason, the positional identity of slices was maintained during hippocampal dissection and placement on the culture insert membrane. Quantitative analysis of sprouting confirmed this septal-temporal gradient of sprouting (data not shown). Because of this gradient, the number of septal, medial, and temporal slices analysed was the same for each treatment group; the number of surviving slices per treatment group was 14 ± 1 for all experiments.

Culture medium and hormone treatment

17β-Estradiol, progesterone and tamoxifen citrate (Sigma) were dissolved in ethanol, ethanol, and water, respectively, to a concentration of 10^{-3} M, and then diluted into culture media to final concentrations of 10^{-10} M, 10^{-7} M, and 10^{-7} M, respectively. Control cultures received an equivalant amount of ethanol (0.1% final concentration). Hormone treatment began at one DIV.

Timms's heavy metal staining

The Timm's stain procedure was adapted for slice cultures by Zimmer and Gahwiler.[81] Briefly, cultures are treated with 1% Na_2S for 10 min, fixed in 70% ethanol, rehydrated, then developed with a solution of gum arabic, citrate buffer, and $AgNO_3$ for 35–40 min at 26°, followed by washing in cold water. The membrane was cut out of the plastic insert, mounted on gelatin-coated slides, dehydrated in ethanol, followed by clearing in xylene and mounting in Permount. Histological preparations were analysed by quantitation of the Timm's staining.

Quantitation of mossy fiber sprouting

Slices were visualized on an Olympus VanoxT at ×40 magnification and digitized images were captured with an Optronix LX450 video camera using NIH Image version 1.60 software, a public domain software (available at http://www.rsb.info.nih.gov/nihimage/). Gain and offset parameters were set so that no pixels were saturated at either end of the gray-level spectrum, and maintained constant for each experiment's analysis. Mossy fiber sprouting was measured as described by Coltman *et al.*[12] with treatment group blind to the operator. Using NIH Image, the Timm's staining was quantitated in two supra-granular regions, ventral and dorsal (dorsal included the apical region). Optical density was measured in a 100 μm by 50 μm box placed over the three most intensely staining areas within each of the dorsal, ventral, and central hilar regions. The average transmittance within these regions was converted to gray levels by NIH Image such that 100% transmittance = 0 and 0% transmittance = 255. Background transmittance measurements taken in the adjacent hippocampal fissure were subtracted from measurements of the two supra-granular regions. These corrected sprouting region measurements were divided 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.[12] The sprouting indices were analysed by ANOVA with Fisher PLD post hoc test to assess the significance of differences between groups.

Apolipoprotein E immunoreactivity

After 18 DIV, slices were submersion-fixed in 4% paraformaldehyde for 1 h followed by three rinses in Tris-buffered saline (TBS). Slices were either whole-mounted onto poly-L-lysine-coated slides or 12 μm sections were cut on a Zeiss cryostat. Endogenous peroxidase activity was suppressed with 0.5% H_2O_2 in methanol for 15 min at room temperature (RT) and non-specific binding sites blocked with 5% normal goat serum in 3% BSA for 1 h at RT. The ApoE antibody was applied (overnight at 4°C)

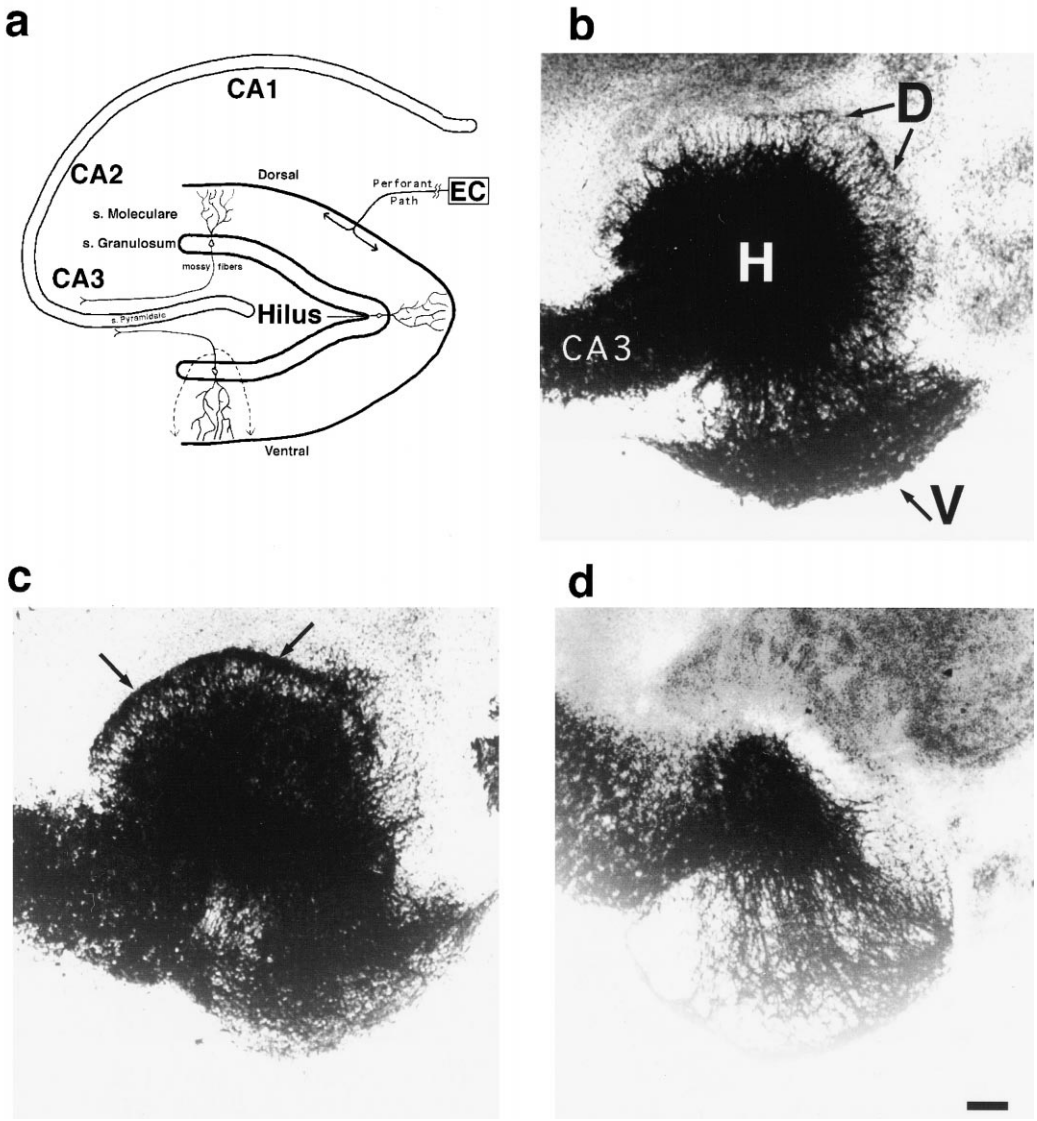

Fig. 1. Mossy fiber sprouting in hippocampal slice cultures. (a) Diagram of hippocampus showing granule cell dendritic tree in the molecular layer with its innervation from the perforant path of the entorhinal cortex (EC), the mossy fibers from the hilus projecting through CA3 to the left, and representations of sprouting mossy fibers indicated by dashed lines in the ventral region. Timm's-stained OHSC after 18 DIV (with CA3 projecting to the left) derived from C57Bl/6J (b); C57Bl/6J treated with 17β-estradiol (10^{-10} M) (c); or ApoE-KO (d). H, hilus; D, dorsal sprouting region; V, ventral sprouting region. Scale bar = 100 μm.

to slices at a 1:800 dilution in TBS containing 0.1% Tween-20, 3% BSA and 8 mM sodium azide. Slices were then rinsed and incubated with a biotinylated anti-mouse secondary antibody (1:500 in TBS plus Tween-20; 1 h at RT). Slices were rinsed and incubated with avidin–biotin–peroxidase complex solution (Elite ABC kit, Vector Labs) for 45 min at RT. Diaminobenzidine-metal enhanced chromagen (Pierce) was used to reveal ApoE antibody binding.

RESULTS

Mossy fiber sprouting in wild-type mice organotypic hippocampal slice culture

Timm's stained mossy fibers showed typical supragranular sprouting after 18 DIV. Sprouting fibers originating from the hilar region projected through the granule cell layer to the molecular layer in the dorsal, apical, and ventral regions (Fig. 1a, b). It is notable that the Timm's stained fiber sprouting in the ventral blade of the dentate was more extensive, projecting throughout the whole molecular layer. Sprouting was quantitated by normalization to Timm's staining in the hilar region.[12] The resulting sprouting index was significantly higher in the ventral region than in the dorsal (Table 1); however, ventral sprouting levels were less than the highest levels reported, which reached 0.9.[12]

Table 1. Quantitative analysis of Timm's-stained mossy fiber sprouting

Strain, treatment	Dorsal		Ventral	
	mean	S.D.	mean	S.D.
C57BL6/J				
Control	0.35	0.04	0.60	0.08
Estradiol	0.68*	0.06	0.66	0.04
Estradiol + progesterone	0.40**	0.08	n.d.	n.d.
Progesterone	0.44	0.09	n.d.	n.d.
Estradiol + tamoxifen	0.40**	0.15	n.d.	n.d.
Tamoxifen	0.35	0.06	n.d.	n.d.
ApoE-KO				
Control	< 0.01		0.54	0.06
Estradiol (10^{-10} M)	< 0.01		0.50	0.08

Fourteen slices per treatment group were analysed; control, 0.1% ethanol; estradiol, 10^{-10} M; progesterone, 10^{-7} M; tamoxifen, 10^{-7} M.

*$P < 0.01$ compared to control; **$P < 0.05$ compared to estradiol alone; n.d., not determined.

Effect of lack of mouse apolipoprotein E expression on mossy fiber sprouting

In contrast to hippocampal slice cultures derived from the background strain, C56Bl/6J (Fig. 1b), cultures derived from ApoE-KO mice did not show Timm's-stained sprouting of mossy fibers into the supragranular region of the dorsal molecular layer (Fig. 1d), even in cultures maintained to 28 DIV (data not shown). The pattern of sprouting in ApoE-KO was limited to the ventral region (V, in Fig. 1b). This pattern was observed in over 250 slices from at least 20 animals from four litters of ApoE-KO. Quantitative analysis showed no difference in the degree of ventral sprouting in ApoE-KO compared to C57Bl/6J (Table 1). These results show that sprouting in the dorsal region was ApoE-dependent while that in the ventral region was ApoE-independent.

Effect of hormones on mossy fiber sprouting

17β-Estradiol treatment (100 pM) increased sprouting in the dorsal region (Fig. 1c, arrows). Quantitative analysis showed that sprouting was significantly increased by 75% (Table 1). Progesterone (100 nM) had no effect by itself, but blocked the 17β-estradiol stimulation (Table 1). The 17β-estradiol antagonist, tamoxifen, also blocked the 17β-estradiol stimulation (Table 1). The ventral region sprouting was not affected by 17β-estradiol in either C57Bl/6J or ApoE-KO (Table 1). The lack of sprouting in the dorsal region of ApoE-KO was not restored by 17β-estradiol treatment (Table 1).

Apolipoprotein E immunoreactivity in hippocampal slice cultures

The pattern of ApoE immunoreactivity in hippopcampal slice cultures was determined after 18 DIV. ApoE immunoreactivity was found fairly uniformly distributed throughout the hippocampal regions; staining was slightly higher in the stratum oriens and stratum radiatum regions around CA1 (Fig. 2) extending through the stratum pyramidale in CA3 and dentate gyrus, and slightly lower in the stratum moleculare. The majority of immunoreactive cells appeared to be astrocytes (Fig. 2, inset arrows).

DISCUSSION

Although ApoE deficiency does not compromise PNS regeneration, perhaps by compensatory overproduction of another apolipoprotein,[55] it appears to be essential in the CNS.[37,38,52] Results shown here indicate that ApoE is required for sprouting of mossy fibers to the OML in the dorsal DG in response to EC deafferentation in OHSC. This is consistent with the impaired synaptic regeneration in ApoE-KO in response to entorhinal cortex deafferentation *in vivo*.[38]

ApoE has many activities which could account for its CNS effects, with cholesterol and phospholipid metabolism[36] consistently implicated in its role in CNS and PNS plasticity after injury.[5,33] A model for this role has been described[5,52,53,59] where glia phagocytosing degenerating terminals esterify cholesterol from scavenged membrane lipid, repackaged it with ApoE and secrete it, facilitating delivery of cholesterol to neurons via their ApoE receptors for neurite growth. In support of this model, (i) ApoE synthesis increases in the deafferented ML after ECL[53,54] possibly due to up-regulation by intracellular free cholesterol and oxidized low-density lipoprotein (LDL);[7,66,67] (ii) ApoE-KO fails to clear degenerating terminals in the ML after ECL,[17] an activity attributed to both astrocytes and microglia;[3] (iii) ApoE-KO and LDL receptor-knockout mice show altered synaptic plasma membrane cholesterol distribution and phospholipid content,[29] a phenomenon also observed in aged wild-type mice;[30] and (iv) ApoE stimulates neurite outgrowth.[16,48,56,73a]

The regional restriction of the defective sprouting response may reflect the differences in developmental age between dorsal and ventral DG. Granule cell mossy fibers in the dorsal DG develop earlier (beginning at PD3) than in the ventral region,[35,65] and the granule cell terminal field in the ML as well as its entorhinal innervation develop earlier in the dorsal DG.[18,34,65] Developmental age of granule cells and their mossy projections may explain differential mossy fiber sprouting observed in the hippocampus septal-temporal axis.[12,34,57,69] Since 85% of granule cells are born postnatally and the mossy fiber system develops unusually slowly over the first three weeks postnatally,[20,65] the large increase in ApoE expression at this time[15,44,46] could play an important role in determining the consequences of neurodegeneration. Our results showing that mossy fiber sprouting

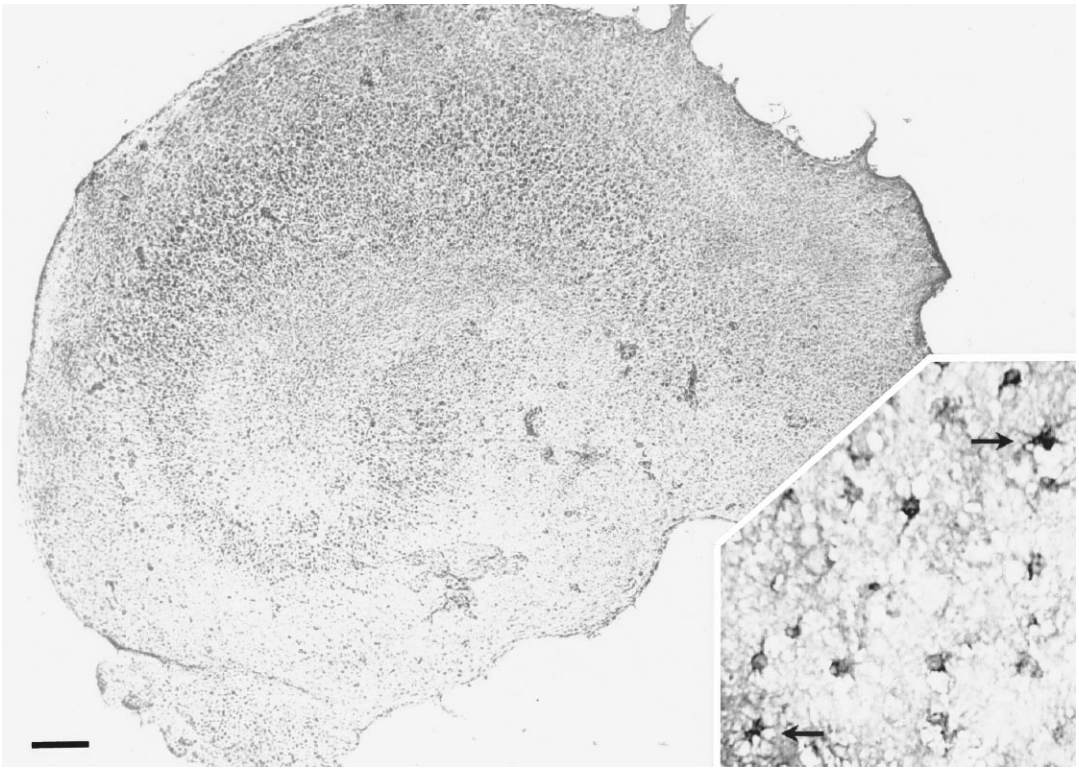

Fig. 2. ApoE immunoreactivity in hippocampal slice culture. ApoE immunoreactivity of a whole mount of a hippocampal slice at 18 DIV shows overall diffuse staining. At higher power, immunoreactivity of a 12 μm section of a hippocampal slice in the stratum pyramidale layer in CA1 shows cellular staining with astrocytic morphology (inset; arrows). Scale bar = 200 μM.

in the older, dorsal DG is ApoE-dependent, while sprouting in the younger, ventral DG in ApoE-independent, may reflect changes in hippocampal plasticity during development that represent a developmental age-dependent change in the capability of the granule cells to respond to deafferentation or an age-dependent change in the underlying mechanism of sprouting.

Sprouting shows an age-dependent reduction in response to ECL[62] which models aspects of EC neurodegeneration in AD.[23] Aged humans show increased intra- and supra-granular Timm's staining, suggesting that the ML might be partially deafferented with age.[9] Although some AD patients have shown aspects of regenerative sprouting in the DG,[23] whether compensatory growth is widespread and whether it reverses cognitive deficits has been debated, since functional recovery requires that reactive synaptogenesis not exacerbate circuitry dysfunction.[14,39,52]

AD prevalence is increased in women, and postmenopausal estrogen replacement therapy reduces AD risk,[27,49] delays onset,[74a] and improves cognition in both AD and non-AD.[64] Among estrogen's numerous CNS effects, neural-organizational effects include enhanced neurite sprouting, which has been shown for several brain regions in *in vivo*, organotypic, and *in vitro* systems.[26,43,76] Our results of estrogen stimulating sprouting of mossy fibers in OHSC extend these studies to sprouting that has a functional compensatory effect in a neuronal population pathologically relevant to AD.

Estrogen effects can be either receptor- or non-receptor-mediated. Estrogen-induced sprouting in OHSC is probably receptor-mediated since the estrogen receptor antagonist, tamoxifen, which has been shown to block estrogen-induced neuritogenesis in hypothalamic neurons,[11] blocked estrogen's effect. In addition, progesterone also blocked estrogen-induced sprouting, consistent with previous reports in which progesterone blocks estrogen's induction of hippocampal dendritic spine density.[80]

It is not known whether these effects are due to direct action of estrogen on neurons, which could occur at several levels: activational,[79] trophic,[4,11,76] and protective (including anti-oxidant and anti-inflammatory).[11] Alternatively, indirect effects include those on glia. Estrogen up-regulation of ApoE synthesis in glia[71,75] and stimulation of sprouting in OHSC is consistent with the model of glia repackaging cholesterol with ApoE to support new neuronal membrane synthesis (as described above). In addition, our results in OHSC are supported by recent studies *in vivo* where compensatory synaptogenesis (synaptophysin immunoreactivity) in the hippocampus in response to EC lesion is both stimulated by estrogen

replacement in ovariectomized animals and is defective in ApoE-KO animals.[70] The role of ApoE in estrogen-stimulated sprouting in OHSC is supported by the regional coincidence of their effects: (i) sprouting in the dorsal DG was both ApoE-dependent and stimulated by estrogen; (ii) ApoE-independent sprouting in the ventral DG was not affected by estrogen; and (iii) estrogen did not restore sprouting in the dorsal DG of ApoE-KO, suggesting that ApoE expression is required for estrogen's effect on sprouting (see note added in proof). It was recently shown that up-regulation of ApoE synthesis in glia (primarily astrocytes) occurs in CNS regions that undergo estrus cycle-dependent synaptic remodeling.[71]

CONCLUSION

Together, these results indicate an important interaction between estrogen and ApoE in synaptic plasticity. Although it is not known whether estrogen stimulation of sprouting is relevant to AD, its regulation of ApoE expression in glial cells may, in part, contribute to enhancing compensatory sprouting leading to cognitive improvement.

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Note added in proof—The difference in regional sensitivity of granule cells to estrogen-stimulated neurotrophic effects has also been observed in adult, female rats, where short-term estrogen replacement in long-term estrogen-derived females increases dentate granule cell spine density primarily by the dorsal region. [G. Einstein, personal communication; Miranda P., Williams C. L., and Einstein G. Granule cells are sexually dimorphic in their response to estradiol (submitted)].