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# Regulation of hippocampal cholesterol metabolism by apoE and environmental stimulation

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**Abstract**

Alzheimer's disease is associated with genetic risk factors, of which the allele E4 of apolipoprotein E (apoE4) is the most prevalent, and it is also affected by environmental factors such as early life education. We have recently shown, utilizing apoE-deficient and apoE transgenic mice, that synaptogenesis in the hippocampus following environmental stimulation is affected by apoE. In view of the pivotal role of cholesterol in synaptic plasticity, and of its suggested role in synaptogenesis, we presently examined the effects of apoE and environmental stimulation on brain cholesterol homeostasis. The hippocampal levels of cholesterol and its precursors and metabolites in control mice were not affected by exposure to environmental stimulation. In contrast, the hippocampal levels of cholesterol and its precursors lathosterol and desmosterol and metabolite 24S-hydroxycholesterol were lower in apoE-deficient mice that were maintained in a regular environmental than those of corresponding control mice, whereas they were markedly elevated following environmental stimulation. Histological and immunohistochemical experiments revealed that the combined stimulatory effects of apoE deficiency and environmental stimulation on cholesterol metabolism were associated with marked activation of hippocampal astrocytes and with the abnormal accumulation of cholesterol in neurons and astrocytes. These effects were rescued similarly in apoE3 and apoE4 transgenic mice. These findings suggest that apoE plays an important role in the translocation of cholesterol from astrocytes to neurons *in vivo* and in the regulation and homeostasis of this process.

**Keywords:** Alzheimer's disease, ApoE, astrocytes, cholesterol, enriched environment, synaptic plasticity.
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Alzheimer's disease (AD) is associated with genetic risk factors, of which the allele E4 of apolipoprotein E (apoE4) is the most prevalent (Corder *et al*. 1993; Saunders *et al*. 1993; Roses 1996). However, its pathology is also affected by environmental factors, including low levels of education at childhood and an impoverished socio-economic background (Zhang *et al*. 1990; Katzman 1993; Kawas *et al*. 1999; Moceri *et al*. 2000).

We have recently shown that the phenotypic expression of the apoE genotype is affected by environmental factors. More specifically, exposure of young mice transgenic to human apoE3, that is the AD benign apoE allele, and exposure of control and apoE-deficient mice to environmental stimulation (van Praag *et al*. 2000) results in improved learning and memory and also stimulates synaptogenesis (Levi *et al*. 2003; Ophir and Levi 2004). In contrast, the learning and memory of the apoE4 transgenic mice and their hippocampal synapses are not affected by exposure to environmental stimulation (Levi *et al*. 2003, 2005). The mechanism by which apoE4 impairs hippocampal plasticity and, isoform specifically, blocks the environmental stimulation of synaptogenesis and memory is presently not known.

Neurite outgrowth *in vitro* is stimulated by cholesterol but even more effectively by apoE and cholesterol-containing lipoproteins (Handelmann *et al*. 1992). In addition, neurite

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*Abbreviations used*: AD, Alzheimer's disease; apoE4, the allele E4 of apoliporotein E; GFAP, glial fibrillary acidic protein; PBS, phosphate-buffered saline.

 

outgrowth is blocked by inhibitors of cholesterol synthesis (Michikawa and Yanagisawa 1998, 1999; Michikawa *et al.* 2000). This suggests that apoE and cholesterol play important and interacting roles in neurite outgrowth. Furthermore, neurite outgrowth *in vitro* is a stimulated isoform specifically by apoE3, whereas apoE4 is either inhibitory or has no effect (Nathan *et al.* 1994; Bellosta *et al.* 1995; Holtzman *et al.* 1995; Nathan *et al.* 1995; DeMattos *et al.* 1998). Neuronal synaptogenesis *in vitro* is also stimulated by apoE in a cholesterol-dependent manner (Mauch *et al.* 2001). It is, however, not yet known whether synaptogenesis is affected isoform specifically by apoE. Furthermore, the secretion of cholesterol by astrocytes is apoE dependent and is greater in astrocytes that express apoE3 than in the corresponding apoE4 astrocytes (Nathan *et al.* 1994; Pitas *et al.* 1998; Gong *et al.* 2002).

Co-culture studies suggest the occurrence of a cholesterol shuttle from astrocytes to neurons that, upon demand, supplies neurons with cholesterol at levels higher than they can synthesize (Pfrieger 2003). Accordingly, astrocytic cholesterol that is secreted together with apoE is taken up and utilized by the neurons. A fraction of the neuronal cholesterol is metabolized to 24S-hydroxycholesterol (24S-OHChol) and is released and subsequently taken up by astrocytes, where it stimulates cholesterol efflux (Pfrieger 2003; Bjorkhem and Meaney 2004). The role of the astrocytes to neuron cholesterol shuttle in cholesterol homeostasis and neuronal plasticity *in vivo* is not known.

We presently investigated the role of apoE and cholesterol in synaptic plasticity. In addition to cholesterol, we analysed lanosterol, desmosterol and lathosterol, which are steroidal precursors in the mevalonate pathway and whose levels reflect the rate of cholesterol synthesis, as well as the ubiquitous and brain-specific cholesterol metabolites cholestanol and 24S-hydroxycholesterol. This was performed by measurements of the effects of environmental stimulation on cholesterol metabolism and on astrocytic activation in brains of control and apoE-deficient mice and of apoE3 and apoE4 transgenic mice.

## Materials and methods

### Transgenic mice

Human apoE3 and apoE4 transgenic mice were generated on an apoE-deficient C57BL/6J background utilizing human apoE3 and apoE4 transgenic constructs as previously reported (Xu *et al.* 1996). The experiments were performed with the apoE3-453 and apoE4-81 lineages that express similar levels of brain apoE (Levi *et al.* 2003). The apoE transgenic mice were back bred with genetically homogenous apoE-deficient mice (Jackson Laboratories catalogue no. N10JAX) for more than 10 generations and were heterozygous for the human apoE transgene and homozygous for mouse apoE deficiency. The apoE genotype of the mice was confirmed by PCR analysis as previously described (Levi *et al.* 2003). The control animals were C57BL/6J.

### Environmental stimulation

Three-week-old apoE3 and apoE4 transgenic mice, along with control and apoE-deficient mice with the same background (C57BL/6J), were placed for 24 weeks in either regular cages or in cages that contained exploratory objects such as toys, tunnels and a running wheel (van Praag *et al.* 2000) ($n = 5$ males per mouse group in each of the environments).

### Preparation of brain homogenates

The brain samples examined are from the same mice whose learning and memory and synaptic plasticity, as well as the associated effects thereon of environmental stimulation, are described in (Levi *et al.* 2003). Briefly, control apoE-deficient and apoE3 and apoE4 transgenic mice were killed after exposure to the indicated environment for 24 weeks during the last 4 weeks of which their learning and memory performances were tested (Levi *et al.* 2003). The mice were anaesthetized with ketamine and killed by transcardial perfusion with phosphate-buffered saline (PBS), after which the brains were removed and halved. The hippocampus and cortex of the left hemisphere were homogenized in 20 mM Tris HCl pH 8.0 that contained 140 mM NaCl, 10% glycine and 1% NP40 and protease inhibitors (Calbiochem, San Diego, CA, USA), after which they were aliquoted and stored frozen at −70°C. The right brain hemisphere was fixed and used for the histological experiments as previously described (Levi *et al.* 2003).

### Determination of cholesterol-related lipids

Cholesterol, cholesterol precursors and metabolites were extracted from the hippocampal and cortical homogenates with yields greater than 90% as previously described (Lutjohann *et al.* 2002). Levels of cholesterol were determined by gas-liquid chromatography – flame ionization detector with 5α-cholestane as the internal standard. The levels of cholesterol precursors (lathosterol, lanosterol, desmosterol) and the cholesterol metabolite cholestanol were determined by gas-liquid chromatography – mass spectrometry, using epicoprostanol as the internal standard, as previously described (Lutjohann *et al.* 2002), whereas the cholesterol metabolite 24S-hydroxycholesterol (24S-OHChol) was determined by gas-liquid chromatography – mass spectrometry using [$^2H_4$]24OHChol as internal standard. The levels of cholesterol and the related sterols are presented as per whole hippocampus and cortex, as are the corresponding protein levels. Protein was determined by the bicinchoninic acid method utilizing bovine serum albumin as the standard.

### Immunohistochemistry and histochemistry

Frozen brain coronal sections (25 μm) were cut at the level of the anterior hippocampus (2 mm posterior to Bregma). The sections were stained immunohistochemically with an anti-glial fibrillary acidic protein (GFAP) primary mAb (dilution 1 : 1000; Pharmagen, San Jose, CA, USA) and a biotinylated goat anti-mouse secondary antibody (dilution 1 : 1000; Jackson Immuno-Research, West Grove, PA, USA) as previously described (Ophir *et al.* 2003). The immunostained brain sections were viewed and photographed at a magnification of ×10 using a Supercam camera (Applitec Ltd, Holon, Israel). Cholesterol was stained by incubation for 2 h with

filipin complex (100 μg/mL; Sigma, St Louis, MO, USA) in PBS which contained 0.05% Triton X 100 followed by 3 × 5-min rinses in PBS. The bound filipin was then visualized as previously described (Yamada *et al*. 2001). Double labelling for GFAP and filipin was performed by pre-staining the cells with anti-GFAP (dilution 1 : 1000, Pharmagen) and Cy-3-labelled fluorescent second antibodies. The fluorescently stained brain sections were viewed and photographed at the indicated magnification using a Nikon camera.

### Immunoblot assays

Immunoblot analysis was performed by loading equal amounts of the hippocampal homogenates (10 μg protein/lane) onto 12% polyacrylamide gels that were then subjected to electrophoresis and blotted. GFAP was detected and visualized with an anti-GFAP mAb (dilution 1 : 1000; Pharmagen) and HRP-labelled goat anti-mouse secondary antibodies (dilution 1 : 1000; Jackson Immuno-Research). The intensities of the immunoblot bands were quantified by computerized densitometry (Levi *et al*. 2003).

### Statistical analysis

The values of cholesterol and of the related lipids of each of the groups × treatment are expressed as the mean ± SD and are normalized relative to the corresponding value of control mice that were maintained in a regular environment. Differences among means of the experimental groups (four mouse groups × two treatments) were analyzed by two-way ANOVA with treatment and genotype as the independent factors. Post-hoc comparisons of the results were performed when ANOVA showed a significant difference, using a Fisher LSD tests for multiple comparisons.

## Results

### ApoE deficiency and brain cholesterol metabolism

The effects of apoE deficiency and environmental stimulation on hippocampal cholesterol levels are presented in Fig. 1(a). As shown, the hippocampal cholesterol levels of apoE-deficient mice that were maintained in regular cages were lower than those of wild-type control mice. In contrast, the hippocampal cholesterol levels of apoE-deficient mice that were exposed to the enriched environment were markedly higher than those of control mice, whose corresponding cholesterol levels were not affected by environmental stimulation (Fig. 1a). ANOVA of the cholesterol results revealed a significant effect of group × treatment ($p < 0.001$). Further analysis revealed that the hippocampal cholesterol levels of apoE-deficient mice that were maintained in regular and enriched environments (60 ± 10 and 170 ± 10%, respectively, of the regular control) differed significantly from each other ($p < 0.001$), as well as from the cholesterol levels of control mice ($p < 0.002$).

Next, we investigated the extent to which the observed effects of apoE deficiency and environmental stimulation on hippocampal cholesterol are related to alteration in cholesterol synthesis. This revealed that the hippocampal levels of the cholesterol precursors lathosterol and desmosterol of

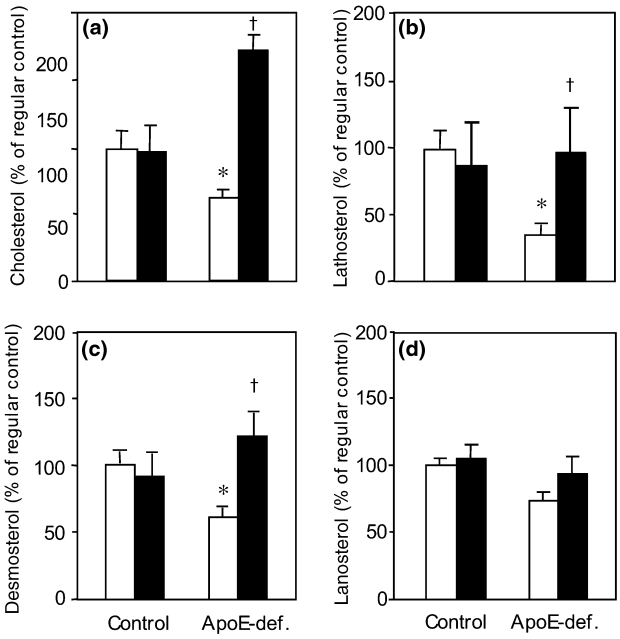


**Fig. 1** The effects of apoE and environmental stimulation on hippocampal levels of cholesterol (a), lathosterol (b), desmosterol (c) and lanosterol (d). Control and apoE-deficient (ApoE-def) mice were subjected to either regular (white bars) or enriched (black bars) environments after which the mice were killed and their hippocampal cholesterol, lathosterol, lanosterol and desmosterol levels were determined as described in Materials and methods. Results presented (mean ± SD of five mice in each of the four groups) were each normalized relative to the corresponding values of control mice that were maintained in a regular environment (100% = 214 ± 26 μg cholesterol, 0.35 ± 0.04 μg lathosterol, 0.88 ± 0.04 μg desmosterol, and 0.02 ± 0.01 μg lanosterol per hippocampus. *$p < 0.001$ for comparison of the cholesterol, lathosterol and desmosterol levels of apoE-deficient and control mice that were maintained in a regular environment; †$p < 0.002$, $p < 0.01$ and $p < 0.004$ for the effect of environmental stimulation on the cholesterol, lathosterol and desmosterol levels, respectively, of apoE-deficient mice.

apoE-deficient mice that were maintained in the regular environment were lower than those of the corresponding control, and that environmental stimulation elevated the lathosterol and desmosterol levels of the apoE-deficient mice, but had no effect on those of control mice (Figs 1b and c) ($p < 0.01$ for lathosterol and $p < 0.001$ for desmosterol for group × treatment by ANOVA). Accordingly, the lathosterol and desmosterol levels of apoE-deficient mice that were maintained in the regular environment were significantly lower than those of corresponding control mice (40 ± 10 and 61 ± 6%, respectively, of regular control; $p < 0.001$), and were markedly elevated by environmental stimulation to 97 ± 30 and 120 ± 12%, respectively, of regular control ($p < 0.01$ and $p < 0.001$ for the effects of environmental stimulation on the hippocampal lathosterol and desmosterol levels, respectively, of apoE-deficient mice). In contrast, the

level of lanosterol, that is the first cholesterol precursor in steroid form and is further upstream than the other precursors in the cholesterol biosynthetic pathways, was not significantly affected by either apoE or environmental stimulation (Fig. 1d).

The effects of apoE and environmental stimulation on cholesterol catabolism in the hippocampus were monitored by measuring their effects on the levels of the cholesterol metabolites 24S-OHChol and cholestanol. As shown in Fig. 2(a), the level of 24S-OHChol in the hippocampus of apoE-deficient mice that were maintained in a regular environment was lower than that of the corresponding control mice. Furthermore, the 24S-OHChol level of apoE-deficient mice was elevated by environmental stimulation to a level similar to that of control mice, whose 24S-OHChol level was not affected by environmental stimulation ($p < 0.001$ for group × treatment by ANOVA). Further analysis revealed that the 24S-OHChol level of the apoE-deficient mice that were maintained in the regular environment was significantly lower than that of the corresponding control mice (25 ± 10%; $p < 0.001$) and that it was elevated by environmental stimulation to 90 ± 10% of the regular control ($p < 0.001$). In contrast, the hippocampal cholestanol level of apoE-deficient mice that were maintained in a regular environment was higher than that of control mice (Fig. 2b). Environmental stimulation resulted in further elevation in the hippocampal cholestanol level of the apoE-deficient mice, but in no change in that of the controls (Fig. 2b; $p < 0.001$ for group × treatment by ANOVA and $p < 0.002$ for the effect of environmental stimulation on the cholestanol levels of the apoE-deficient mice).

The hippocampal protein content of control mice increased from 2.32 ± 0.08 to 3.2 ± 0.8 mg per hippocampus following environmental stimulation, whereas the corresponding protein contents of the apoE-deficient mice were not changed (2.64 ± 0.16 and 2.56 ± 0.08 mg per hippocampus, respectively). These effects however, were not significant ($p > 0.3$ by ANOVA regarding the effects of group, treatment and group × treatment).

The brain area specificity of the effects of apoE and environmental stimulation on cholesterol metabolism was investigated by measuring their effects on cortical cholesterol metabolism. This revealed that, unlike in the hippocampus, the cortical levels of cholesterol, lathosterol, desmosterol and 24S-OHChol of apoE-deficient mice which were maintained in a regular environment were similar to those of the corresponding controls (Fig. 3). As was observed in the hippocampus, the cortical levels of lanosterol were not affected, whereas the cholestanol levels were slightly elevated by apoE deficiency (not shown). Environmental

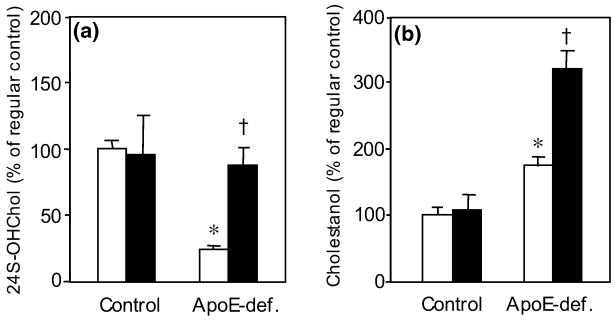


**Fig. 2** The effects of apoE and environmental stimulation on hippocampal levels of the cholesterol metabolites 24S-OHChol (a) and cholestanol (b). Control and apoE-deficient (ApoE-def) mice were subjected to either regular (white bars) or enriched (black bars) environments after which the mice were killed and their hippocampal 24S-OHChol and cholestanol levels were determined as described in Materials and methods. The 24S-OHChol and cholestanol results presented (mean ± SD of five mice in each of the four groups) were normalized relative to the corresponding values of control mice that were maintained in a regular environment and for which 100% = 1.2 ± 0.02 μg/24S-OHChol and 0.4 ± 0.06 μg cholestanol per hippocampus. *$p < 0.001$ for comparison of the 24S-OHChol and cholestanol levels of apoE-deficient and control mice that were maintained in a regular environment; †$p < 0.001$ for the effects of environmental stimulation on apoE-deficient mice.

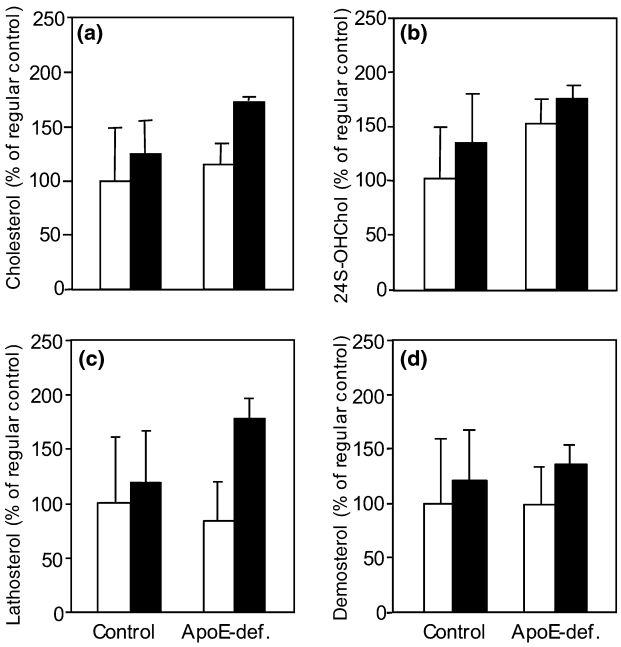


**Fig. 3** The effects of apoE and environmental stimulation on the cortical levels of cholesterol, 24S-OHChol, lathosterol and desmosterol, control and apoE-deficient (ApoE-def.) mice were subjected to either regular (white bars) or enriched (black bars) environments after which the mice were killed and their cortical cholesterol, 24S-OHChol and cholestanol levels were determined as described in Materials and methods. Results presented (mean ± SD of five mice in each of the four groups) were normalized relative to the corresponding values of control mice that were maintained in a regular environment for which 100% = 0.36 ± 0.18 μg cholesterol, 1.8 ± 0.74 μg 24S-OHChol and 0.64 ± 0.38 μg lathosterol and 1.8 ± 0.5 μg desmosterol per cortex.

stimulation increased the cortical levels of cholesterol in the apoE-deficient mice. This effect, however, was smaller than that observed in the hippocampus (compare Figs 1 and 3).

Measurements of the effects of apoE and environmental stimulation on plasma cholesterol levels revealed, in accordance with previous observations (Williamson *et al*. 1992), that the plasma cholesterol levels of apoE-deficient mice that were maintained in a regular environment (985 ± 280 μg/mL; $n = 5$) were markedly higher than those of control (60 ± 18 μg/mL) mice and that environmental stimulation markedly lowered the plasma cholesterol levels of the apoE-deficient mice (450 ± 180 μg/mL), but had no effect on that of control mice (63 ± 10 μg/mL).

### ApoE deficiency and the cellular distribution of cholesterol

The effects of apoE and environmental stimulation and the cellular and spatial distribution of cholesterol in the hippocampus was investigated histochemically utilizing the cholesterol binding and fluorescence compound filipin. As shown in Fig. 4, the intensity of filipin staining in the hippocampus of apoE-deficient mice which were maintained in a regular environment was markedly lower than that of the corresponding controls. Furthermore, filipin staining in the hippocampi of apoE-deficient mice increased dramatically following environmental stimulation to levels higher than those of control mice, whose corresponding levels of filipin binding were not affected by environmental stimulation (Figs 4 and 5). This effect was pronounced in both the neurites and cell bodies of the hippocampal granular and pyramidal neurons and was strongest in the hippocampal dentate gyrus and CA1 subfields (Figs 4 and 5). Furthermore, filipin staining in the perikarya was associated with perinuclear cholesterol 'hot spots', whose levels in the apoE-deficient mice increased dramatically following environmental stimulation. The levels of the perinuclear cholesterol 'hot spots' of control mice were not affected by environmental stimulation and were slightly higher than those of apoE-deficient mice which were kept in the regular environment.

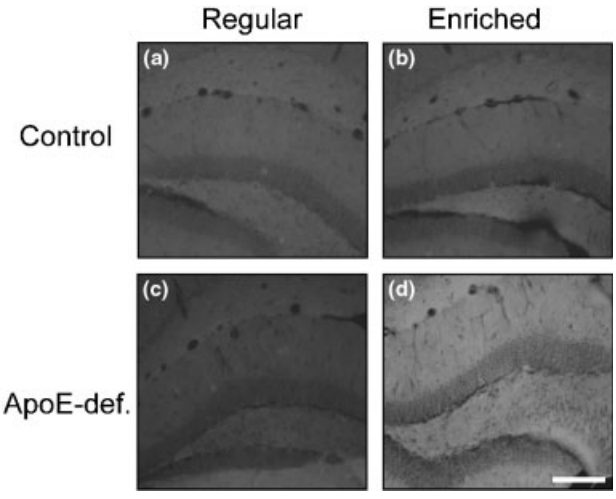


**Fig. 4** The effects of apoE and environmental stimulation on the levels and spatial distribution of cholesterol in the hippocampus. Cholesterol was visualized histochemically utilizing the fluorescence dye filipin as described in Materials and methods. Pictures shown were photographed at a magnification of ×10 and are representative of coronal sections of control (a, b) and apoE-deficient mice (c, d) which were exposed to regular (a, c) and enriched (b, d) environments. Bar = 200 μm.

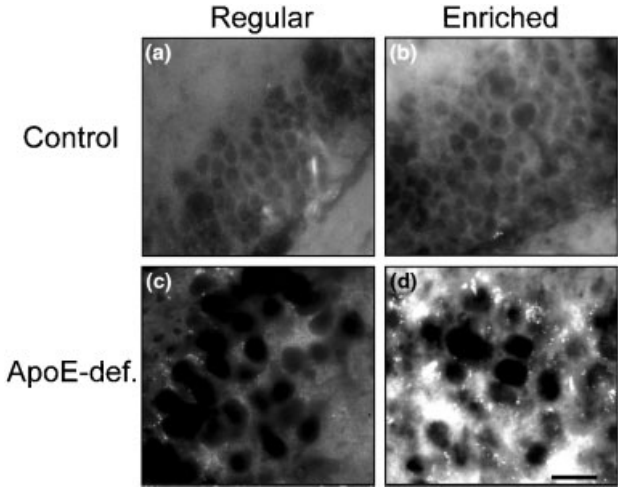


**Fig. 5** Neuronal localization of the elevated levels of cholesterol in apoE-deficient and control mice following environmental stimulation. Pictures shown were photographed at a magnification of ×60 and are representative coronal sections of control (a, b) and apoE-deficient mice (c, d) which were exposed to regular (a, c) and enriched (b, d) environments. Filipin staining was performed as described in Materials and methods. Bar = 50 μm.

The extent to which elevation of the hippocampal cholesterol content of environmentally stimulated apoE-deficient mice was also associated with the accumulation of cholesterol in astrocytes was examined by double labelling the sections with filipin and the astrocytic marker GFAP. As can be seen in Fig. 6, the hippocampus of the environmentally stimulated apoE-deficient mice contained a large number of GFAP-positive cells, a fraction of which (~15%) also contained filipin-positive cholesterol 'hot spots'. The levels of GFAP-positive astrocytes was markedly lower in apoE-deficient and control mice, which were kept in a regular environmental, and in environmentally stimulated control mice, and the few GFAP-positive cells which were seen in these mice did not contain the filipin-positive 'hot spots'. Additional immunohistochemical experiments revealed that environmental stimulation was associated with a marked increase in the number of GFAP-positive hippocampal astrocytes of apoE-deficient mice, whereas the corresponding control astrocytes were not so affected (Fig. 7). Furthermore, immunoblot assays revealed that

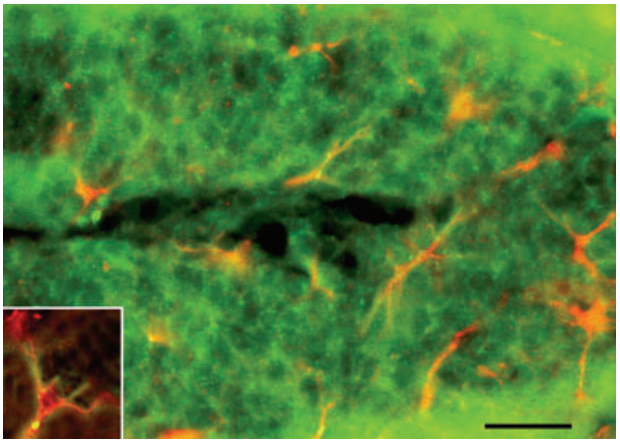

**Fig. 6** Astrocytic localization of elevated levels of cholesterol in environmentally stimulated apoE-deficient mice. Coronal hippocampal sections were double labelled with the cholesterol dye filipin and for the astrocytic marker GFAP, as described in Materials and methods. The filipin and GFAP staining are shown in green and red, respectively, whereas the areas in which they co-localize are depicted in yellow. Section shown was photographed at a magnification of ×40 and is from the tip of the dentate gyrus hippocampal subfield of a representative brain. Insert depicts an activated astrocyte with cholesterol droplets at a 2-fold higher magnification. Bar = 50 μm.

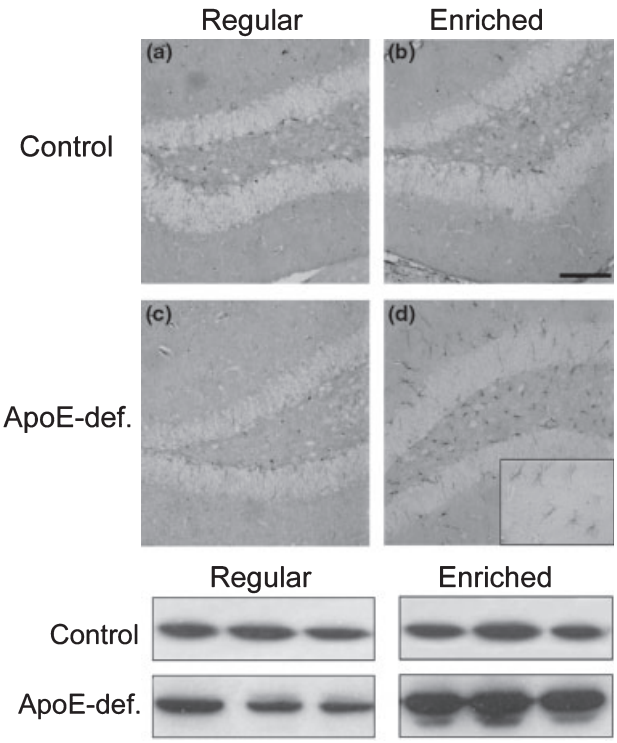


**Fig. 7** The effects of apoE and environmental stimulation on the levels of activation of hippocampal astrocytes. The upper panels correspond to GFAP immunohistochemical stains of representative coronal sections of control (a, b) and apoE-deficient mice (c, d) that were maintained in regular (a, c) and enriched environments (b, d). The lower panels depict GFAP immunoblots of three representative mice of each of the groups. The GFAP immunohistochemistry and immunoblots assays were performed as described in Materials and methods. Bar = 200 μm. Insert depicts activated astrocytes at a 4-fold higher magnification.

control and apoE-deficient mice, that were maintained in the regular environment, had similar levels of hippocampal GFAP (93 ± 30% of regular control) and that environmental stimulation markedly elevated the GFAP level of apoE-deficient mice to 252 ± 50% of regular control but had no effect on that of the controls (Fig. 7).

Taken together, these findings suggest that environmental stimulation of apoE-deficient mice is associated with marked accumulation of cholesterol in hippocampal neurons and with the activation of hippocampal astrocytes and the accumulation of cholesterol in these cells.

### ApoE transgenic mice and brain cholesterol metabolism

The extent to which the effects of apoE deficiency on brain cholesterol metabolism and astrocytic activation can be rescued by either apoE3 or apoE4 was investigated, utilizing transgenic mice which express these human apoE isoforms on a null mouse apoE background. As shown in Table 1, the hippocampal levels of cholesterol and of its metabolites, lathosterol, desmosterol and 24S-OHChol of the apoE3 and apoE4 transgenic mice were similar and, like those of the controls, were not significantly affected by environmental stimulation. Furthermore, unlike in the apoE-deficient mice, the levels of these sterols in the apoE3 and apoE4 transgenic mice do not differ significantly from those of the controls. The hippocampal cholestanol levels were, however, affected by the apoE genotype and were higher under regular conditions, but not following environmental stimulation, in the apoE4 than in apoE3 and control mice (Table 1). Furthermore, the levels of hippocampal filipin staining and GFAP-positive astrocytes of the apoE3 and apoE4 transgenic mice were both similar to those of control mice and were not affected by environmental stimulation (not shown).

These findings show that the main features of apoE deficiency are rescued by both apoE3 and apoE4. It should, however, be noted that the standard deviation of the data obtained from the apoE4 mice under regular conditions was larger than those of the other mice groups (Table 1). It is thus possible that, in addition to the similar ability of apoE3 and apoE4 to rescue the phenotype of apoE deficiency, they also have more subtle isoform-specific effects on hippocampal cholesterol metabolism. This possibility and the mechanism underlying the isoform-specific effects of apoE on hippocampal cholestanol levels remain to be investigated.

## Discussion

This study revealed that the hippocampal cholesterol metabolism and homeostasis are tightly linked to apoE, and that

**Table 1** The effects of apoE3 and apoE4 and environmental stimulation on hippocampal cholesterol metabolism

| | Regular environment | | | Enriched environment | | |
|---|---|---|---|---|---|---|
| | Control | ApoE3-tg | ApoE4-tg | Control | ApoE3-tg | ApoE4-tg |
| Cholesterol | 100 ± 12 | 90 ± 5 | 103 ± 40 | 97 ± 17 | 84 ± 15 | 71 ± 12 |
| Lathosterol | 100 ± 13 | 75 ± 10 | 92 ± 41 | 84 ± 32 | 75 ± 7 | 67 ± 16 |
| Desmosterol | 100 ± 7 | 84 ± 5 | 95 ± 17 | 95 ± 12 | 80 ± 7 | 76 ± 4 |
| Lanosterol | 100 ± 5 | 95 ± 3 | 102 ± 9 | 102 ± 10 | 97 ± 2 | 94 ± 6 |
| 24S-OHChol | 100 ± 6 | 76 ± 6 | 126 ± 32 | 98 ± 22 | 90 ± 10 | 74 ± 3 |
| Cholestanol | 100 ± 15 | 115 ± 18 | 202 ± 42 | 108 ± 26 | 88 ± 15 | 83 ± 22 |

Control, ApoE3 transgenic (ApoE3-tg) and ApoE4 transgenic (ApoE4-tg) mice were subjected to either regular or enriched environments as described in Materials and methods. The results presented (average ± SD of five mice per group) were normalized relative to the corresponding values of control mice that were maintained in a regular environment and for which 100% = 214 ± 26 μg cholesterol, 0.35 ± 0.04 μg lathosterol, 0.88 ± 0.04 μg desmosterol, 0.02 ± 0.01 μg lanosterol, 1.2 ± 0.02 μg 24S-OHChol and 0.4 ± 0.06 μg cholestanol per hippocampus. $^*p < 0.001$ for the effect of environmental stimulation on the cholesterol levels of the apoE4-tg mice.

the levels of cholesterol and its metabolites in apoE-deficient but not in control mice are markedly affected by environmental stimulation. Accordingly, the hippocampal levels of cholesterol and of its precursors lathosterol and desmosterol, and of its metabolite 24S-OHChol, were lower in apoE-deficient mice that were maintained in a regular environment, than those of control mice, whereas they were markedly higher than those of the controls in environmentally stimulated apoE-deficient mice (Figs 1 and 2). The levels of the cholesterol metabolite cholestanol were affected differently by apoE and were higher in apoE-deficient mice, particularly following environmental stimulation, than in the controls. Histological and immunohistochemical experiments revealed that the combined stimulatory effects of apoE deficiency and environmental stimulation on cholesterol metabolism were associated with marked and abnormal accumulations of cholesterol in neurons and astrocytes (Figs 4,5 and 6) and with hippocampal astrogliosis (Fig. 7).

Almost all brain cholesterol is a product of local *de novo* synthesis by oligodendrocytes, that lay down the cholesterol-rich myelin sheaths, and of astrocytes and neurons (Dietschy and Turley 2004). Furthermore, *in vitro* studies identified a cholesterol shuttle from astrocytes to neurons, that is mediated by apoE (Mauch *et al*. 2001). It has been suggested that the astrocytic cholesterol shuttle can be regulated via a neuronal negative feedback signal that reflects the level of neuronal cholesterol and that such a mechanism may be mediated by 24S-OHChol that is produced in neurons and can inhibit astrocytic cholesterol synthesis (Lund 1999; Pfrieger 2003; Bjorkhem and Meaney 2004).

Astrogliosis and brain apoE levels are elevated following exposure of mice to neuronal insults, as well as by enhanced neuronal activity (Soffie *et al*. 1999) and during ageing (Masliah *et al*. 1996; Soffie *et al*. 1999; Emmerling *et al*. 2000; Seitz *et al*. 2003). Furthermore, apoE deficiency is associated with marked neurodegeneration during ageing and following brain injury and neuronal stress (Masliah *et al*. 1995; Chen *et al*. 1996). These findings suggest that the activity and physiological impact of the astrocytic cholesterol shuttle is enhanced under conditions of neuronal repair and stimulated growth. Because astrocytes have neurotransmitter receptors (Haydon 2001), this could be mediated via neurotransmitters. A schematic presentation of the astrocytic cholesterol shuttle and of its neuronal regulation are presented in Fig. 8.

All together, the present findings suggest that the coupling between the neuronal and astrocytic cholesterol metabolism in control mice is tightly regulated in such a way that environmental stimulation increases the turnover of the cholesterol shuttle without affecting the relative abundance of the cholesterol precursors and catabolites (Figs 1 and 2). Furthermore, the observation that the total hippocampal cholesterol content of control mice is not affected by environmental conditions further suggests that only a small fraction of the total hippocampal cholesterol of control mice turns over. This situation changes markedly in apoE-deficient mice. Accordingly, the hippocampal cholesterol levels of apoE-deficient mice under regular conditions are markedly lower than those of the corresponding control mice and were elevated following environmental stimulation (Figs 1, 2, 4, 5 and 6).

*In vitro* studies revealed that apoE stimulates the accumulation of cholesterol by neurons and that the release of lipoproteins and cholesterol from astrocytes is impaired by apoE deficiency (Fagan *et al*. 1999). This suggests that the translocation of cholesterol from astrocytes to neurons is impaired in apoE-deficient mice under regular conditions. The cholesterol precursors lathosterol and desmosterol and its metabolite 24S-OHChol are established markers of cholesterol synthesis (Lutjohann and von Bergmann 2003; Heverin *et al*. 2004). Accordingly, the findings that the levels of these cholesterol metabolites are also decreased in apoE-deficient mice under regular conditions suggest that the histochemically and biochemically observed decrease in the

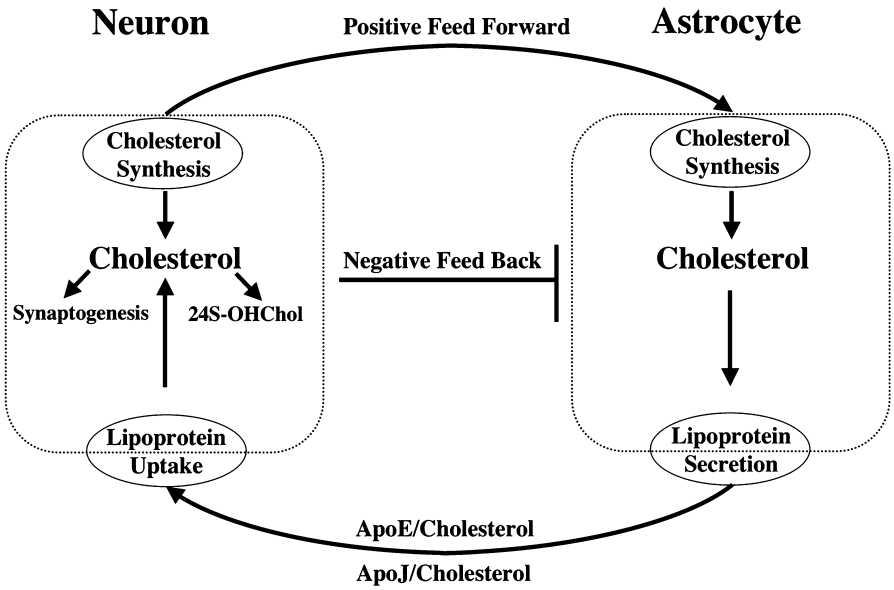


**Fig. 8** A model based on the present *in vivo* results and on previous *in vitro* studies of the metabolic interactions between astrocytes and neurons and of the regulatory effects of apoE and neuronal activity on cholesterol homeostasis in the hippocampus (Pfrieger 2003; Bjorkhem and Meaney 2004). Neuronal cholesterol can be synthesized *in situ* or taken up from the external medium as either apoE- or apoJ-containing lipoproteins that have been secreted from the astrocytes. Increased neuronal activity results in the release of a neuronal factor that then activates the synthesis and secretion of cholesterol by the astrocytes. This may be achieved by neurotransmitters and their interactions with astrocytic neurotransmitter receptors (Haydon 2001). The cholesterol metabolite 24S-OHChol, which is produced only in neurons and can inhibit cholesterol synthesis (Pfrieger 2003; Bjorkhem and Meaney 2004), provides a feedback mechanism via which neurons with excess cholesterol can signal the astrocytes to turn down the cholesterol shuttle. Accordingly, the reduction in cholesterol levels in apoE-deficient mice under regular conditions is related to the breakdown of the cholesterol shuttle as a result of the decrease in astrocytic cholesterol efflux and neuronal cholesterol uptake. Two major changes occur in these mice under stimulatory conditions: (i) the astrocytes are activated and secrete higher levels of cholesterol which, under these conditions, can be taken up by the neurons via an apoE-independent mechanism; (ii) loss of the negative neuronal regulation of astrocytic cholesterol levels.

hippocampal cholesterol levels of these mice is as a result both of impaired translocation of cholesterol from astrocytes to neurons and of decreased cholesterol synthesis. The molecular mechanisms underlying the inhibitory effects of apoE deficiency on cholesterol synthesis under these conditions remain to be determined. The levels of the cholesterol metabolite 24S-OHChol, which is produced only in neurons, are affected more markedly by apoE deficiency under regular conditions than are those of cholesterol and its precursors (Figs 1 and 2), which suggests that the decrease in hippocampal cholesterol levels under these conditions is because of a marked decrease in available neuronal cholesterol.

The findings that environmental stimulation of apoE-deficient mice is associated with enhanced astrocytic activation and with increased levels of astrocytic and neuronal cholesterol are because of the concurrent stimulation and disregulation of the cholesterol shuttle under these conditions. The finding that the levels of the cholesterol precursors also increased under these conditions suggests that the elevation in hippocampal cholesterol levels is related to increased cholesterol synthesis. Furthermore, the metabolic observation that levels of cholesterol metabolite cholestanol, which unlike 24S-OHChol is also produced in astrocytes, are elevated more markedly than 24S-OHChol in environmentally stimulated apoE-deficient mice, suggests that the active cholesterol metabolic pool under these conditions is astrocytic. ApoE deficiency does not completely block cholesterol efflux and some cholesterol is secreted from the astrocytes as apoJ-containing lipoproteins (DeMattos *et al*. 2001; Gong *et al*. 2002; Hirsch-Reinshagen *et al*. 2004). Accordingly, it is possible that observed increase in neuronal cholesterol of environmentally stimulated apoE-deficient mice is as a result of apoJ-mediated translocation of cholesterol from astrocytes into the neurons. This interpretation is also consistent with the finding that the largest elevation in hippocampal cholesterol levels following prolonged environmental stimulation is neuronal. The elevation in neuronal cholesterol levels could also be because of *in situ de novo* synthesis. However, as the levels of the neuron-specific metabolite 24S-OHChol of the apoE-deficient mice are elevated less markedly by environmental stimulation than the corresponding cholesterol precursors (Figs 1 and 2), and in view of the marked astrocytic activation which is observed under these conditions, the increase in neuronal cholesterol levels is probably as a result

mainly of the uptake of secreted astrocytic cholesterol. Another key feature of the system under these conditions of apoE deficiency and environmental stimulation is the apparent dysfunction of the negative feedback neuronal inhibitory mechanisms which relates the levels of neuronal cholesterol to the astrocytes (Fig. 8). This suggests that there are apoE-dependent mechanisms, in addition to 24S-OhChol, which under these conditions can down-regulate the astrocytic cholesterol shuttle. It has been proposed that apoE also plays a role in the reverse translocation of cholesterol away from neurons (Sloop *et al*. 1987; Fagan *et al*. 1999). It is therefore possible that the increased levels of neuronal cholesterol in environmentally stimulated apoE-deficient mice are also related to impairments under these conditions in the ability of the neurons to remove excess cholesterol.

Cholesterol homeostasis in the cortex, unlike in the hippocampus, is not markedly affected by apoE deficiency (compare Fig. 1 and Figs 2–3). This is in accordance with the observation that the cortex contains lower levels of astrocytes than the hippocampus (San Jose *et al*. 2001), and suggests that the apoE-dependent astrocytic cholesterol shuttle has a less robust impact on cholesterol homeostasis in the cortex than in the hippocampus. It should be noted, however, that the present study was performed with whole cortex homogenates, and that it is therefore possible that distinct cortical regions are differentially affected by apoE. The plasma cholesterol levels of apoE-deficient mice were higher than those of controls for mice that were maintained both in regular and enriched environments and, unlike in the brain, were lowered by environmental stimulation. This is probably a consequence of the higher physical activity and metabolic rates associated with environmental stimulation, and is in accordance with the observation that circulatory peripheral cholesterol levels do not reflect the actual cholesterol level in the brain (Williamson *et al*. 1992).

The effects of apoE deficiency and environmental conditions on hippocampal cholesterol levels are rescued similarly by both apoE3 and apoE4 (Table 1). This suggests that the basic effects of apoE on hippocampal neuronal and astrocytic cholesterol homeostasis during environmental stimulation are not apoE isoform-specific. However, it is possible that apoE4 has more subtle, isoform-specific effects on hippocampal cholesterol metabolism which are apparent under distinct pathological conditions, such as high cholesterol diets.

Environmental stimulation induces an increase in synaptogenesis and synaptic plasticity in the hippocampus of control and apoE-deficient mice (Levi *et al*. 2003). These experiments and the presently reported cholesterol metabolism studies were performed with the same lines of mice and under similar environmental conditions. This suggests that synaptic plasticity in young apoE-deficient mice under regular conditions and following gradual and prolonged exposure to environmental stimulation, is not affected by disregulation of the astrocytic cholesterol shuttle. Neuronal plasticity and repair of apoE-deficient mice are however, impaired following acute insults such as in head injury (Chen *et al*. 1997; Han and Chung 2000). It is therefore possible that the synaptogenesis-related physiological impacts of the astrocytic cholesterol shuttle are apparent under conditions of such acute stimuli. Further evidence for the possible dissociation between synaptogenesis and cholesterol metabolism is provided by the apoE4 transgenic mice, whose hippocampal cholesterol metabolism is similar to those of control and apoE3 transgenic mice (Table 1), but which, unlike these mice, do not undergo enhanced hippocampal synaptogenesis following environmental stimulation (Levi *et al*. 2003, 2005). The possible role of additional mechanisms, such as β-amyloid-related cross-talk interactions between the environmental stimulation apoE genotype (Lazarov *et al*. 2005) or altered signal transduction (Herz and Bock 2002) in mediating the effects of apoE4 on hippocampal plasticity, remains to be determined.

In conclusion, the present findings show that apoE plays an important role in the translocation of cholesterol from astrocytes to neurons *in vivo* and in the regulation and homeostasis of this process.

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