Amyloid- β protein modulates the perivascular clearance of neuronal apolipoprotein E in mouse models of Alzheimer's disease

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Abstract The deposition of amyloid- β protein (A β) in the brain is a hallmark of Alzheimer's disease (AD). Apolipoprotein E (apoE) is involved in the clearance of A β from brain and the *APOE* ϵ 4 allele is a major risk factor for sporadic AD. We have recently shown that apoE is drained into the perivascular space (PVS), where it co-localizes with A β . To further clarify the role of apoE in perivascular

clearance of A β , we studied apoE-transgenic mice over-expressing human apoE4 either in astrocytes (GE4) or in neurons (TE4). These animals were crossbred with amyloid precursor protein (APP)-transgenic mice and with APP-presenilin-1 (APP-PS1) double transgenic mice. Using an antibody that specifically detects human apoE (h-apoE), we observed that astroglial expression of h-apoE in GE4 mice leads to its perivascular drainage, whereas neuronal expression in TE4 mice does not, indicating that neuron-derived apoE is usually not the subject of perivascular drainage. However, h-apoE was observed not only in the PVS of APP-GE4 and APP-PS1-GE4 mice, but also in that of APP-TE4 and APP-PS1-TE4 mice. In all these mouse lines, we found co-localization of neuron-derived h-apoE and A β in the PVS. A β and h-apoE were also found in the cytoplasm of perivascular astrocytes indicating that astrocytes take up the neuron-derived apoE bound to A β , presumably prior to its clearance into the PVS. The uptake of apoE-A β complexes into glial cells was further investigated in glioblastoma cells. It was mediated by α_2 macroglobulin receptor/low density lipoprotein receptor-related protein (LRP-1) and inhibited by adding receptor-associated protein (RAP). It results in endosomal A β accumulation within these cells. These results suggest that neuronal apoE-A β complexes, but not neuronal apoE alone, are substrates for LRP-1-mediated astroglial uptake, transcytosis, and subsequent perivascular drainage. Thus, the production of A β and its interaction with apoE lead to the pathological perivascular drainage of neuronal apoE and provide insight into the pathological interactions of A β with neuronal apoE metabolism.

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Introduction

The amyloid- β protein ($A\beta$) is a major component of Alzheimer's disease (AD)-related amyloid plaques (Masters et al. 1985) and its soluble aggregates are presumably the driving force behind AD-related neurodegeneration (Gotz et al. 2001; Kaye et al. 2003; Lewis et al. 2001; Oddo et al. 2006; Shankar et al. 2008). Increasing $A\beta$ levels lead to the development of senile plaques in AD mouse models over-expressing amyloid precursor protein (APP) (Games et al. 1995; Hsiao et al. 1996; Sturchler-Pierrat et al. 1997), eventually also leading to neuron loss in mice expressing APP and presenilin 1 (APP-PS1) (Schmitz et al. 2004). Elevated $A\beta$ levels in the brain can result from increased production and from insufficient clearance (Iwata et al. 2000; Miller et al. 2003; Weller et al. 2008). Increased production of $A\beta$ is mainly restricted to the familial forms of AD, which are caused by mutations in either *APP* (Chartier-Harlin et al. 1991; Goate et al. 1991; Murrell et al. 1991), presenilin-1 (*PSEN1*) (Sherrington et al. 1995) or presenilin 2 (*PSEN2*) genes (Levy-Lahad et al. 1995; Rogaev et al. 1995). On the other hand, sporadic AD is thought to result from decreased clearance of $A\beta$ from brain. Several mechanisms have been proposed for $A\beta$ clearance: (1) drainage into the perivascular space (PVS) (Weller et al. 2008), (2) enzymatic degradation by neprilysin and/or insulin-degrading enzyme (IDE) (Iwata et al. 2000; Miller et al. 2003), (3) active transport across the blood-brain barrier (BBB) (Bell and Zlokovic 2009; Deane et al. 2008), and (4) endocytosis by microglia and astrocytes (Akiyama et al. 1996; Funato et al. 1998; Thal et al. 2000; Yamaguchi et al. 1998). Apolipoprotein E (apoE) is involved in the perivascular drainage of $A\beta$ (Thal et al. 2007) and in its clearance across the BBB (Bell and Zlokovic 2009; Deane et al. 2008). The *APOE* $\epsilon 4$ allele is a major risk factor for sporadic AD (Strittmatter et al. 1993a) and the gene dosage of *APOE* $\epsilon 4$ has a strong influence on the age of AD onset (Corder et al. 1993; Ghebremedhin et al. 2001). Because AD cases with capillary cerebral amyloid angiopathy (capCAA) are strongly associated with the *APOE* $\epsilon 4$ allele (Thal et al. 2010), we speculate that alterations in apoE-related clearance of $A\beta$ play a major role in sporadic AD. ApoE as well as apoE- $A\beta$ complexes are taken up via lipoprotein receptors such as the α_2 macroglobulin receptor/low density lipoprotein receptor-related protein (LRP-1) (Beffert et al. 1998; Koistinaho et al. 2004; Williams et al. 1997). LRP-1 is expressed in neurons and astrocytes (Moestrup et al. 1992; Thal et al. 1997). The function of LRP-1 can be inhibited by its receptor-associated protein (RAP) (Williams et al. 1992).

To further clarify the role of apoE in $A\beta$ clearance, we studied two strains of transgenic mice over-expressing

human apoE4 (h-apoE), either driven by the astroglia-specific GFAP promoter (GE4) or by the neuron-specific Thy-1-promoter (TE4) (Van Dooren et al. 2006). These mice were crossbred with APP[V717I] and APP[V717I]-PS1[A246E]-transgenic mice (Van Dooren et al. 2006). The two types of apoE-transgenic mice differ only in the cell-type expression of h-apoE, but otherwise express similar levels of h-apoE (Van Dooren et al. 2006). In APP[V717I]-PS1[A246E]-TE4 and APP[V717I]-PS1[A246E]-GE4-transgenic mice, over-expression of h-apoE results in increased levels of soluble $A\beta$ and CAA, but decreased cortical plaque load in comparison to APP[V717I]-PS1[A246E] mice (Van Dooren et al. 2006). The analysis using a h-apoE-specific antibody allowed us to trace the fate of neuron-derived and astroglia-derived h-apoE separately under near-physiological conditions in TE4 and GE4 mice as well as under AD-related conditions in APP[V717I]-TE4, APP[V717I]-GE4, APP[V717I]-PS1[A246E]-TE4 and APP[V717I]-PS1[A246E]-GE4 mice. Comparative analysis of these six genotypes of mice is, therefore, ideally suited to study the interaction between apoE and $A\beta$ and its role in $A\beta$ clearance. Our results suggest that glia limitans astrocytes are responsible for the apoE-related perivascular clearance of $A\beta$ and, moreover, that $A\beta$ modulates the clearance of neuronal apoE into the PVS.

Materials and methods

Animals

Fifteen months old female TE4 ($n = 4$), GE4 ($n = 4$), APP[V717I]-TE4 ($n = 4$), APP[V717I]-GE4 ($n = 4$), APP[V717I]-PS1[A246E]-TE4 ($n = 4$) and APP[V717I]-PS1[A246E]-GE4 mice ($n = 2$) were used for this study. APP[V717I] ($n = 3$) and APP[V717I]-PS1[A246E] mice ($n = 2$) were used as controls. All mice were hemizygous for the transgenes expressed and with an identical mixed FVB/N, C57BL6 genetic background (Van Dooren et al. 2006). The TE4 mice express human *APOE* $\epsilon 4$ driven by the neuron-specific Thy-1 promoter (Tesseur et al. 2000) and the GE4 mice express it driven by the astrocyte-specific GFAP promoter (Tesseur et al. 2000). The APP[V717I] mice express human *APP* gene carrying the London mutation [V717I] under the control of Thy-1 promoter (Moechars et al. 1999). The APP[V717I]PS1[A246E] mice additionally express mutant [A246E] human *PSEN1* driven by the Thy-1 promoter and produce more $A\beta$ -deposits than APP[V717I] mice (Dewachter et al. 2000). We used APP[V717I]-TE4 and APP[V717I]-GE4 mice for testing the effects of glia- and neuron-derived apoE on cellular $A\beta$ -uptake and its perivascular clearance. APP[V717I]-PS1[A246E]-TE4 and APP[V717I]-PS1[A246E]-GE4 mice were used to confirm

the results in a second model. All animals were handled according to the directives of the ethical commission for animal welfare of the Catholic University of Leuven.

Cell culture and A β -uptake with and without blocking of LRP-1 function

LN229 glioblastoma cells were used for studying apoE–LRP-1 related uptake of A β . These cells were chosen because glioblastoma cells have astrocytic properties (Kleihues et al. 2007). Cells were cultured in DMEM supplemented with 10% fetal calf serum. Incubation with A β _{1–40} (Peptides International, Louisville, KY, USA) diluted in DMEM in a 15 μ M concentration was performed at 37°C in the incubator. In parallel, LN229 glioblastoma cells were incubated with 15 μ M A β _{1–40} and 0.5 μ M RAP (BioMac, Leipzig, Germany) to inhibit the apoE-mediated uptake of A β via lipoprotein receptors such as LRP-1. After 12 h, the incubation medium was removed and the cells were fixed in 4% paraformaldehyde for immunocytochemistry. This experiment was repeated twice.

Immunoprecipitation of apoE from cell culture medium of LN229 glioblastoma cells

Conditioned medium of untreated LN229 cells was collected over 3 days to demonstrate secretion of apoE into the medium. For immunoprecipitation, 1 ml of the conditioned medium was incubated with anti-h-apoE (D6E10, Covance, Princeton, NJ, USA, 1/500) for 5 h under gentle agitation at 4°C. Protein G-coated microbeads (Milenyi Biotec, Bergisch-Gladbach, Germany) were added to the sample for overnight incubation at 4°C. The mixture was then passed through the μ Columns, which separate the microbeads by retaining them into the column, while the rest of the lysate flows through. After few mild washing steps, the microbead-bound proteins were eluted with 1 $\times$ LDS sample buffer at 90°C (Invitrogen, Carlsbad, CA, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the eluted proteins was carried out and western blotting with anti-h-apoE (D6E10, Covance, 1/1,000) was performed as previously described (Utter et al. 2008).

Neuropathology and immunohistochemistry

For fixation and immunohistological analysis of the brain, mice were deeply anesthetized and transcardially perfused with ice-cold saline. The brain was rapidly excised and one hemisphere was fixed in phosphate-buffered 4% paraformaldehyde overnight at 4°C. Free floating 40 μ m sagittal sections were cut with a vibrating microtome and stored in phosphate-buffered saline (PBS; pH 7.5) containing 0.1%

sodium azide at 4°C until further use. Endogenous mouse apoE (m-apoE) was immunohistologically detected with a goat polyclonal antibody directed against m-apoE (polyclonal goat; Santa Cruz, Santa Cruz, CA, USA; 1/100, microwave and formic acid pre-treatment), and the transgenic h-apoE was detected with a mouse monoclonal antibody (D6E10; Covance, 1/500, microwave and formic acid pre-treatment). The D6E10 antibody does not significantly cross-react with the m-apoE (Thal et al. 2007). For detection of A β , an antibody raised against A β _{17–24} (4G8; Covance, Princeton, NJ, USA; 1/5,000) or a rabbit polyclonal antibody raised against A β _{1–42} [3,552, polyclonal rabbit, 1/1,000 (Page et al. 2010)] were used with formic acid pre-treatment. For staining astrocytes, an antibody raised against glial fibrillary acidic protein (GFAP; polyclonal rabbit; DAKO, Glostrup, Denmark; 1/1,000) was used. The apoE-receptor LRP-1 (CD-91) was detected with a monoclonal antibody (α 2-M-R-II2C7; BioMac, Leipzig, Germany; 1/150; microwave and protease pretreatment) and/or with a polyclonal antibody [LRP-1704; polyclonal rabbit antibody (Pietrzik et al. 2002), 1/50, microwave pretreatment]. Early endosomal antigen (EEA) was observed with a polyclonal antibody (rabbit polyclonal, Developmental Studies Hybridoma Bank, University of Iowa, USA, 1/200). To block endogenous mouse immunoglobulin G (IgG) when working with mouse primary antibodies or to detect endogenous IgG, sections were incubated with unlabeled polyclonal goat anti-mouse IgG (polyclonal goat; Biomedica, Foster City, CA, USA; 1/75) either prior to incubation with mouse primary antibodies, as reported previously (Thal et al. 2007), or as a primary antibody. For permeabilization, sections were incubated with 0.25% Triton-X, 1.82% DL-Lysine and 10% bovine serum albumin in 10 mM Tris buffer (TBS; pH: 7.5) for 90 min at 22°C. For immunohistochemical staining, endogenous peroxidase activity was quenched by incubating the sections in 2% H₂O₂ for 30 min at 22°C. Biotin labeled secondary antibodies were used in conjugation with Vectastain ABC elite kit (Vector; Peterborough, UK) as the tertiary reagent and developed with 3,3-diaminobenzidine-HCl (Sigma–Aldrich; St. Louis, MO, USA) or Vector peroxidase kit SG (Vector). For fluorescence immunostaining, secondary antibodies directly labeled with Cy2 and Cy3 (Jackson Immunoresearch; Suffolk, UK; 1/50) were used. Briefly, sections were washed with TBS, and followed by permeabilization and blocking were incubated with appropriate primary antibodies diluted in TBS for 16–24 h at 22°C. Unbound antibody was removed by TBS washes and sections were incubated with appropriate secondary antibodies for 2 h at 22°C. After washing, sections were then mounted on SuperFrost Plus slides (Menzel-Gläser; Braunschweig, Germany) and dried at 37°C for 30 min, followed by a brief dip in xylene and then mounted

with Eukitt (O. Kindler; Freiburg, Germany). For visualization and documentation, Leica DMLB (Filters: Y3 for Cy3 and GFP CY2 for Cy2) microscopes as well as a Leica TCS NT confocal laser scanning microscope (Leica, Bensheim, Germany) were used. Stacks of 2D images were superimposed digitally using the Image J Imaging Processing and Analysis software (NIH, Bethesda, MD, USA), and Z-project data sets were generated for the visualization of astroglial location of h-apoE and A β .

In all mice, we determined dichotomously the presence or absence of m-apoE, and h-apoE in astrocytes, neurons and in the perivascular space. The presence or absence of A β in the PVS was also observed dichotomously. For this purpose, one anti-m-apoE, one anti-h-apoE and one anti-A β -stained sagittal section covering frontal, parietal and occipital cortex, hippocampus, basal ganglia and thalamus were analyzed for each individual mouse. As an exception, sections from GE4 and TE4 mice were not stained with anti-A β because these mice did not express human A β .

Statistical analysis

Statistical analysis was performed using PASW Statistics software (version 18, Chicago, IL, USA). The Fisher exact test was applied to compare TE4, GE4, APP[V717I]-TE4, APP[V717I]-GE4, APP[V717I]-PS1[A246E]-TE4 and APP[V717I]-PS1[A246E]-GE4 mice for the presence or absence of human apoE in the PVS and in perivascular astrocytes. *p* values were appropriately corrected for multiple testing.

Results

The main findings of this study are that under normal conditions, the perivascular drainage of apoE is different for astroglia-derived apoE and neuron-derived apoE and that A β is required to enable astroglial uptake and perivascular drainage of neuron-derived apoE. Astroglial uptake of A β occurs in the presence of apoE via lipoprotein receptors such as LRP-1 into the early endosomal compartment as demonstrated in cultured glioblastoma cells.

Perivascular drainage and astroglial uptake of astroglia-derived and neuron-derived h-apoE and its relation to A β -clearance in transgenic mouse models

GE4 mice exhibited h-apoE in the cortical and perivascular astrocytes as well as in the PVS (Fig. 1, Table 1). Although most neurons lacked h-apoE in GE4 mice, some cortical and hippocampal neurons contained cytoplasmic h-apoE (Fig. 1f) indicating that h-apoE produced by astrocytes is secreted into the extracellular space and taken up by

neurons. The h-apoE positive neurons were scattered throughout the cortex without an obvious preference. Neurons of the basal ganglia and the thalamus did not exhibit intracytoplasmic h-apoE (data not shown). TE4 mice, on the other hand, showed h-apoE in neurons, but not in PVS and astrocytes (Fig. 1a, c; Table 1). This discrepancy in the occurrence of perivascular and astroglial h-apoE was confirmed to be statistically significant for all investigated GE4 and TE4 mice (Fisher exact test: perivascular h-apoE, *p* = 0.028; astroglial h-apoE, *p* = 0.028). Astroglial and neuronal expression of h-apoE in GE4 and TE4 mice did not lead to differences in the pattern of m-apoE expression. m-apoE occurred in astrocytes, neurons as well as in the PVS of all GE4 and TE4 mice investigated (Fig. 1j, k; Table 1). Plaques were not observed in GE4 and TE4 mice.

The neurons in GE4 and TE4-mice expressed LRP-1 (Fig. 1l, m). Single astrocytes also exhibited LRP-1 (data not shown). These findings confirmed the neuronal and astroglial expression of this receptor in the brain parenchyma described already by other authors (Moestrup et al. 1992; Thal et al. 1997). Taken together, neuron-derived h-apoE was neither cleared into the PVS nor taken up by astrocytes under near-physiological conditions.

In TE4 and GE4 mice cross-bred with APP[V717I] and APP[V717I]-PS1[A246E] mice, we observed h-apoE immunoreactivity not only in the PVS of all APP[V717I]-GE4 (Fig. 2a–c) and APP[V717I]-PS1[A246E]-GE4 mice, but also in that of all APP[V717I]-TE4 (Fig. 2d–f) and APP[V717I]-PS1[A246E]-TE4 mice (Table 1). Comparison between TE4 mice, on the one hand, and APP[V717I]-TE4 and APP[V717I]-PS1[A246E]-TE4 mice, on the other, revealed significant differences in the presence of h-apoE in the PVS (Fisher exact test: h-apoE, *p* = 0.004). In all four APP[V717I]-h-apoE expressing mouse lines, A β was co-localized with h-apoE in the PVS (Fig. 2a–f, Table 1).

h-apoE immunoreactivity was observed in astrocytes of APP[V717I]-GE4, APP[V717I]-PS1[A246E]-GE4, APP[V717I]-TE4 and APP[V717I]-PS1[A246E]-TE4 mice (Fig. 2g–i, Table 1), often also exhibiting intracellular A β (Fig. 2m–r, Table 1). No differences in the occurrence of astroglial h-apoE between the four mouse lines were observed. Comparing APP[V717I]-TE4 and APP[V717I]-PS1[A246E]-TE4 mice with TE4 mice, we noticed a significant difference in the presence/absence of astroglial h-apoE (Fisher exact test: h-apoE, *p* = 0.004, Table 1). h-apoE was also observed in the amyloid plaques of APP[V717I]-GE4, APP[V717I]-TE4, APP[V717I]-PS1[A246E]-GE4 and APP[V717I]-PS1[A246E]-TE4 mice without obvious differences from the A β patterns (Fig. 3a–d). Endogenous m-apoE was also found in amyloid plaques (Fig. 3e–h) as well as in the PVS, astrocytes and neurons in

Fig. 1 **a–f** Expression of h-apoE in h-apoE-transgenic mice is either driven by the neuron-specific Thy-1 promoter (TE4: **a**, **c**) or by the astroglia-specific GFAP promoter (GE4: **b**, **d**, **e**, **f**). TE4 mice expressed h-apoE in neurons and neuronal processes (**a**, **arrows** in **c**). Perivascular h-apoE was not found in these mice (**arrowheads** in **a**). GE4 mice, on the contrary, exhibited h-apoE in astrocytes (**arrows** in **b** and **e**) as well as in the PVS (**arrowheads** in **b** and **d**), indicating that astrocytes are necessary for the normal perivascular drainage of apoE. Most neurons in GE4 mice do not contain h-apoE (**arrowheads** in **e** and **f**). However, some neurons contained h-apoE in the cytoplasm (**arrows** in **f**) within these animals indicating that h-apoE produced by astrocytes can be taken up by neurons under physiological conditions. **g–i** Double-label immunofluorescence for h-apoE and GFAP confirmed the astroglial expression (**arrows** in **g–i**) and perivascular clearance of h-apoE (perivascular apoE does not co-localize with GFAP: **arrowhead** in **g–i**) in GE4 mice. **j** Endogenous m-apoE was detected in TE4 mice in neurons (N), astrocytes (**arrows**) as well as in the PVS (**arrowheads**). **k** In GE4 mice, m-apoE was likewise found in neurons (N), astrocytes (**arrows**) and in the perivascular space (**arrowheads**). **l**, **m** LRP-1 was expressed in cortical neurons of TE4 (**l**) and GE4 mice (**m**). Stainings as indicated. Calibration bar in **i** corresponds to: **a**, **b**: 80 μ m; **c**, **d**, **e**, **f**: 25 μ m; **g–i**: 10 μ m. Calibration bar in **m** corresponds to: **j**, **k**: 50 μ m; **l**, **m**: 25 μ m

APP[V717I]-GE4, APP[V717I]-TE4, APP[V717I]-PS1 [A246E]-GE4 and APP[V717I]-PS1[A246E]-TE4 mice. h-apoE positive, CAA-affected vessels were only occasionally observed in some sections from APP[V717I]-

PS1[A246E]-GE4 and APP[V717I]-PS1[A246E]-TE4 mice (data not shown).

In APP[V717I]-TE4, APP[V717I]-PS1[A246E]-TE4, APP[V717I]-GE4 and APP[V717I]-PS1[A246E]-GE4

Table 1 Occurrence of apoE in astrocytes, neurons and perivascular space of GE4-, TE4-, APP[V717I]-GE4 (APP-GE4)-, APP[V717I]-TE4 (APP-TE4)-, APP[V717I]-PS1[A246E]-GE4 (APP/PS-GE4)- and APP[V717I]-PS1[A246E]-GE4 (APP/PS-GE4)-mice

Mouse-line	GE4 (%)	TE4 (%)	APP-GE4 (%)	APP-TE4 (%)	APP/PS-GE4 (%)	APP/PS-TE4 (%)
Astrocytes						
m-apoE	100.00	100.00	100.00	100.00	100.00	100.00
h-apoE	100.00	0.00	100.00	100.00	100.00	100.00
Neurons						
m-apoE	100.00	100.00	100.00	100.00	100.00	100.00
h-apoE	100.00	100.00	100.00	100.00	100.00	100.00
Perivascular space						
m-apoE	100.00	100.00	100.00	100.00	100.00	100.00
h-apoE	100.00	0.00	100.00	100.00	100.00	100.00
A β	n.d.	n.d.	100.00	100.00	100.00	100.00

The table presents the percentage of the mice with a given genotype to exhibit m-apoE and h-apoE in astrocytes, neurons and the perivascular space, as well as A β in the perivascular space. The presence or absence of m-apoE, h-apoE and A β in astrocytes, neurons and the perivascular spaces was determined dichotomously for each animal

mice as well as in APP[V717I] mice, IgG was evident only inside the lumen of small blood vessels and not in the brain parenchyma (Fig. 3i–k). Thus, the BBB appeared intact in these animals.

LRP-1-dependent uptake of A β in glioblastoma cells

LN229 glioblastoma cells treated with A β _{1–40} showed uptake of A β after 12 h of incubation. A β , thereby, co-localized to EEA indicating that it is present in the early endosomal compartment of the cells (Fig. 4a–c). This uptake was strikingly diminished in the presence of RAP, which inhibited the function of lipoprotein receptors such as LRP-1. Cells treated with A β _{1–40} and RAP did not show significant co-localization of EEA with A β (Fig. 4d–f). Control cells incubated only with medium did not exhibit significant amounts of intracellular A β indicating that intrinsic A β -production is negligible under given experimental conditions (Table 2).

Western blot analysis of cell culture medium from LN229 cells displayed an apoE band (Fig. 4g) confirming earlier studies (Oropeza et al. 1987) that glioblastoma cells as those with astroglial properties (Kleihues et al. 2007) are capable of secreting apoE into the extracellular space. Immunocytochemistry showed that LN229 glioblastoma cells exhibited apoE as well as LRP-1 (Fig. 4h, i). ApoE and LRP-1-positive material was seen in vacuoles in the cytoplasm (Fig. 4h, i; arrows). LRP-1 was also found in a diffuse pattern in the cell membrane and in the cytoplasm of LN229 cells (Fig. 4i, arrowheads).

Discussion

Our results demonstrate that under near-physiological conditions, neuron-derived apoE is not a significant

substrate for perivascular drainage in contrast to astroglial apoE. Only in the event of increased A β levels and extracellular A β -deposits as in the crosses with the APP-transgenic mice, did neuron-derived apoE get cleared into the PVS where it co-localized with A β , as in amyloid plaques. Perivascular astrocytes representing the glia limitans, i.e., the border between the brain parenchyma and the PVS, are responsible for uptake of apoE and A β and their clearance into the PVS.

These interpretations are supported by the following findings:

- (1) TE4 mice expressing h-apoE in neurons do not show h-apoE in the PVS or in astrocytes indicating that neuron-derived h-apoE is neither taken up by astrocytes nor cleared into the PVS under near-physiological conditions (Fig. 5a). In contrast, endogenous m-apoE, which is produced by astrocytes, is cleared into the PVS.
- (2) In both, APP[V717I]-TE4 and APP[V717I]-PS1 [A246E]-TE4 mice with increased A β -levels because of APP[V717I] overexpression, neuron-derived h-apoE is found together with A β in the PVS and in perivascular astrocytes indicating that neuronal h-apoE is taken up by astrocytes only in the presence of A β . The uptake of apoE–A β complexes by glial cells has been confirmed in human LN229 glioblastoma cells. LN229 cells, thereby, secrete apoE, which is taken up again when it is coupled to A β . Here, apoE-receptors such as LRP-1 are responsible for the uptake of such apoE–A β complexes into the endosomal compartment. Similar results were obtained in primary mouse astrocytes by Koistinaho et al. (2004). This uptake was inhibited by RAP, as shown in glioblastoma cells, and by anti-apoE antibodies, as demonstrated by Koistinaho et al. (2004).

Fig. 2 a–c: APP[V717I]-GE4 mice exhibited astroglia-derived h-apoE (arrows in **a**, **c**) together with A β (arrow in **b**, **c**) in the PVS. **v** indicates the vessel lumen. **d–f** h-apoE (arrows in **d**) and A β (arrows in **e**) were also found in co-localization (arrows in **f**) in the PVS of APP[V717I]-PS1[A246E]-TE4 mice. **v** indicates the vessel lumen. **g–i** A few astrocytes detected with an antibody against GFAP (arrows in **g**, **i**) also contained h-apoE (arrows in **h**, **i**) in APP[V717I]-PS1[A246E]-TE4 mice. **j–l** In APP[V717I]-GE4 mice GFAP-positive astrocytes (arrows in **j**, **l**) exhibited h-apoE (arrows in **k**, **l**) because of its GFAP promoter driven expression. **m–o** A GFAP-positive perivascular astrocyte of a APP[V717I]-PS1[A246E]-TE4 mouse (arrow in **m**, **o**) also contained A β (arrow in **n**, **o**). **v** indicates a capillary. **p–r** In a APP[V717I]-PS1[A246E]-GE4 mouse, a GFAP-positive astrocyte (arrow in **p**, **r**) exhibited dot-like A β granules in the cytoplasm (arrow in **q**, **r**). Stainings are as indicated. Calibration bar in **r** corresponds to: **a–c**, **g–l**: 25 μ m; **d–f**: 45 μ m; **m–r**: 13 μ m

Fig. 3 **a–h** Extracellular amyloid plaques exhibited h-apoE (**a–d**) as well m-apoE (**e–h**) in APP[V717I]-TE4 (**a**, **e**), APP[V717I]-GE4 (**b**, **f**), APP[V717I]-PS1[A246E]-TE4 (**c**, **g**) and APP[V717I]-PS1[A246E]-GE4 mice (**d**, **h**). **i–k** APP[V717I] (**i**), APP[V717I]-TE4 (**j**) and APP[V717I]-GE4 (**k**) mice did not exhibit extravascular IgG indicative of an intact BBB. IgG was only found inside vessel lumina (arrows). Stainings are as indicated. Calibration bar in **d** corresponds to: **a–h**: 20 μ m. Calibration bar in **k** corresponds to: **i–k**: 20 μ m

An alternative explanation for the presence of h-apoE and A β in the PVS is that there was leakage through the BBB possibly damaged by CAA. If this assumption is true, one would also expect the reverse, i.e., leakage of plasma proteins into the brain parenchyma. However, as this was not observed, leakage through the damaged BBB to explain the presence of neuron-derived h-apoE in the PVS of APP[V717I]-TE4 and APP[V717I]-PS1[A246E]-TE4 mice is dismissed.

The finding that A β modifies astroglial uptake of neuron-derived apoE and its clearance into the PVS strongly suggest a novel action of A β in modulating the clearance of neuronal apoE (Fig. 5b). Under AD-related conditions in APP[V717I]-TE4 and APP[V717I]-PS1[A246E]-TE4 mice, neuronal apoE is proposed to bind A β , resulting in apoE–A β complexes as previously described in the AD brain (Strittmatter et al. 1993b). These complexes may either develop within neurons and are secreted into the extracellular space or they may arise extracellularly. Then they are taken up by astrocytes to be degraded and/or cleared into the PVS as shown in Fig. 5b, presumably via transcytosis. Since neuron-derived apoE is neither observed to end up in astrocytes nor in the PVS under normal conditions, it appears likely that it is not secreted in significant amounts into the extracellular space under normal conditions.

In glioblastoma cells, LRP-1-related uptake of apoE–A β complexes resulted in an early endosomal accumulation of

A β . The role of apoE and lipoprotein receptors such as LRP-1 in this process was indicated by the inhibition of A β -uptake by RAP that blocks lipoprotein receptor function (Williams et al. 1992). Uptake mechanisms independent of lipoprotein receptors and apoE still functioned in the presence of RAP, but did not allow significant A β -uptake into the early endosomal compartment of the cells. Since LN229 glioblastoma cells expressed endogenous apoE as shown here, receptor blocking was, in our opinion, the best way to demonstrate the essential role of apoE in this process. Thus, it is tempting to speculate that such a mechanism is effective also for intraneuronally produced apoE–A β complexes. Early endosomal targeting would then explain a possible secretion of apoE–A β complexes. A further argument for an important role of apoE–A β complexes in the development of A β -pathology is that early A β -deposits usually co-localize to apoE (Thal et al. 2005). The contribution of the endosomal compartment in the development of A β -pathology is highlighted by the detection of A β in neuronal multivesicular bodies in APP-transgenic animals as well as in AD cases (Takahashi et al. 2002) indicating the degradation of endosomal A β .

Perivascular clearance of neuronal apoE under AD-related conditions is pathological and should be regarded as a diversion from the normal apoE trafficking route. It can result in a neuronal apoE deficit and/or in the turning on of compensatory mechanisms to replenish the loss of neuronal apoE, e.g., by increased production of

Fig. 4 Lipoprotein receptor-mediated uptake of A β into LN229 glioblastoma cells. Immunocytochemistry showed anti-EEA-positive vacuoles of the early endosomal compartment (**a**). Treatment of these cells with 15 μ M A β_{1-40} for 12 h lead to an uptake of A β into the cells (**b**). Double-label analysis with a confocal laser scan microscope revealed co-localization of EEA and A β within a large number of early endosomes (**c**). Parallel inhibition of lipoprotein receptors with 0.5 μ M RAP resulted in a reduced uptake of A β into the cells after 12 h of incubation. Anti-EEA indicated that endocytosis into early endosomes is functional in RAP-treated LN229 cells (**d**). However, A β was found only in single dot-like accumulation at the border of the

cell (**e**). Double-labeling with anti-EEA was not evident (**f**), indicating that A β is taken up by glioblastoma cells via lipoprotein receptors. **g** Immunoprecipitation of medium collected from cultured LN229 cells with subsequent western blotting revealed an apoE band at 34 kDa, indicating that LN229 cells secrete apoE. **h, i**: Immunocytochemistry of untreated LN229 cells exhibited intracellular apoE in cytoplasmic vacuoles (arrows in **h**). A similar pattern of strongly stained intracellular vacuoles was also observed with an antibody directed against LRP-1 (arrows in **i**). This antibody also shows diffuse LRP-1 expression in the cytoplasm and on the cell surface (arrowheads in **i**). Calibration bar in **h** is valid for: **a-f**: 5 μ m, **h, i**: 13 μ m

Table 2 ApoE-, LRP-1 expression and colocalization of A β and early endosomal antigen (EEA) in LN229 glioblastoma cells

Treatment	Colocalization EEA – A β	apoE expression	LRP-1 expression
A β	+	+	+
A β + RAP	not significant	+	+
Only medium	not significant	+	+

Application of synthetic A β to LN229 cells for 12 h resulted in an uptake of A β into the early endosomal compartment of the cells as indicated by colocalization of EEA and A β . Parallel treatment with A β and RAP for the same time lead to an inhibition of A β -uptake indicating the lipoprotein receptors such as LRP-1 are responsible for this uptake in cells with astroglial properties. Intrinsic A β -production by LN229 cells was negligible as seen in controls treated only with medium. Under all conditions, LN229 cells exhibited expression of apoE and LRP-1

apoE by the neurons. In addition, increased uptake of extracellular apoE together with A β (Fig. 4b) may negatively impact on neuronal functions. The presence of

astroglia-derived h-apoE in neurons, as demonstrated in the GE4 mice, indicates that neurons are physiologically capable of taking up apoE from the extracellular space

Cerebral clearance of apoE and its modulation by A β

Fig. 5 Schematic representation of the role of apoE in perivascular drainage (a) and its modulation by A β (b). **a** Under normal conditions, astrocytes produce and secrete apoE into the neuropil (1) and the PVS (*). Neurons are capable of taking up apoE from extracellular space (2A). Neuronal apoE is assumed to play a role in synaptic plasticity (Oh et al. 2010) and it can be degraded lysosomally (3). Since we had no evidence for neuron-derived apoE to be taken up by astrocytes and to be cleared into the PVS, it is unlikely that secretion of neuronal apoE plays a significant role under normal conditions (4A). Re-uptake and perivascular clearance of apoE by astrocytes may also take place (2B). **b**: In the AD mouse models, increased A β levels modulate the clearance of apoE. As under normal conditions, apoE is produced by astrocytes and cleared into the extracellular space (1) and into the PVS (*). Extracellular apoE can either be taken up physiologically by neurons (2A) and astrocytes

(2B) or it can bind to extracellular A β (2C). Such apoE-A β complexes can either aggregate and accumulate together with A β to form amyloid plaques (P) or can be taken up by neurons (2D) and astrocytes (2E) that may degrade them lysosomally (3). Astrocytes may also clear them into the PVS (**). Moreover, intraneuronal A β may also interact with apoE inside the neurons (3A). Such apoE-A β complexes can be released from the neurons into the extracellular space (4B) and accumulate in amyloid plaques (P) or taken up by astrocytes (2E) with subsequent lysosomal degradation (3) or perivascular clearance (**). Since the secretion of apoE by neurons physiologically plays a minor role (4A), it is likely that interactions between neuronal apoE and A β are intraneuronal interactions. Whether apoE-A β complexes can be drained into the PVS by neurons themselves or otherwise independently from astrocytes requires further clarification (?).

secreted by astrocytes presumably via LRP-1 and other lipoprotein receptors.

In this study, we focused on the differentiation between astroglial and neuronal expression of apoE and its contribution to AD-related A β -pathology. As already demonstrated by other authors, apoE effects are also modulated by its isoform. As such, mice expressing glial apoE4 and neuronal APP develop more than tenfold A β plaques than mice expressing similar levels of glial apoE3 and neuronal APP (Holtzman et al. 2000). Likewise, Deane et al. (2008) showed that apoE4-A β complexes are less sufficiently

taken up via lipoprotein receptors such as LRP-1 than apoE3-A β complexes presumably explaining the more prominent accumulation of extracellular A β in APOE ϵ 4 carriers (Schmechel et al. 1993; Thal et al. 2010). Neuronal and astroglial expression of apoE, thereby, influence the regional distribution of A β -pathology, especially that of CAA rather than the quantity and overall expansion of A β -deposition (Van Dooren et al. 2006). Thus, APOE genotype influences quantity and overall expansion of A β -deposition, whereas cell-specific apoE liberation may explain distinct local pattern of A β -deposition in the brain

parenchyma and vessels and may, under experimental conditions as presented here, help to understand the roles of neurons and astrocytes in the AD-related interactions between apoE and A β .

Neurodegeneration in APP-transgenic mice primarily leads to dendrite degeneration with a reduced complexity of the dendritic tree (Capetillo-Zarate et al. 2006; Wu et al. 2004). Interestingly, similar changes were reported in transgenic mice expressing apoE4 compared with those expressing apoE3 (Dumanis et al. 2009). This points toward a failing function of intraneuronal apoE that leads to dendritic changes in apoE4 expressing mice, which is proposed to be related to the dendritic alterations described in APP-transgenic mice (Capetillo-Zarate et al. 2006; Wu et al. 2004), as all these models are presumed to exhibit changes in neuronal apoE metabolism either related to its isoform or to its interaction with A β . This hypothesis is supported by the fact that apoE is present in dendrites and it is suggested to be involved in synaptic plasticity (Oh et al. 2010). The intraneuronal interaction between A β and apoE may further reduce the availability of free dendritic apoE and, thereby, affect synaptic plasticity. Such an interaction between apoE and A β may provide a potential pathomechanism to explain the presumed toxicity of intraneuronal A β (Gouras et al. 2010). Another potential consequence of the loss of neuronal apoE, its astroglial uptake and its perivascular drainage together with A β , is that apoE and A β may competitively occupy the glia limitans clearance and transcytosis capacity for proteins of the extracellular space, resulting in the pathological retention of proteins in the brain. Pericapillary A β deposits, i.e., diffuse A β in the glia limitans near capillaries, might represent such a protein retention problem (Attems et al. 2010). Further arguments in favor of a role of apoE–A β interactions in altering protein clearance from the AD brain are: (1) increase of C-terminal truncated apoE in AD cases (Huang et al. 2001; Utter et al. 2008) indicating increased proteolysis of apoE rather than clearance, (2) accumulation of α_2 macroglobulin, especially in its transformed configuration in the AD brain (Strauss et al. 1992; Thal et al. 1997), which is also a ligand of LRP-1 that competitively takes up apoE–A β complexes (Fuentealba et al. 2010; Koistinaho et al. 2004; Kounnas et al. 1995; Rebeck et al. 1993), (3) knockdown of LRP1 by antisense RNA increased the brain levels of A β (Jaeger et al. 2009), (4) blocking the apoE–A β interaction mitigates its toxicity (Sadowski et al. 2004), and (5) APP[V717F $\pm$]–apoE[–/–] mice display a reduced number of amyloid plaques in comparison with APP-transgenic mice with normal apoE expression (Bales et al. 1997).

Our finding that perivascular astrocytes are required for the apoE-related perivascular/vascular clearance of A β highlights the well-known role of astrocytes in contributing to BBB function in the neurovascular and gliovascular unit (Abbott et al. 2006) and points to its possible importance in

the pathogenesis of AD. Astrocytes are known to take up A β that frequently lacks immunoreactivity with antibodies directed against N-terminal epitopes of A β (Akiyama et al. 1996; Funato et al. 1998; Thal et al. 2000; Yamaguchi et al. 1998). Moreover, A β -containing astrocytes frequently occur in association with diffuse amyloid deposits that often lack immunoreactivity for N-terminal epitopes of A β , but co-localize with apoE, e.g., the fleecy amyloid in the entorhinal cortex and the CA1-subiculum region (Thal et al. 2005; Thal et al. 2000). Thus, the astroglial uptake of A β presumably represents its active clearance/transcytosis into the PVS by these cells and explains the resorption of diffuse A β -deposits such as the fleecy amyloid that disappears in the course of the disease (Thal et al. 1999).

In conclusion, our results demonstrate that A β modifies the clearance of neuronal apoE. This unexpected finding indicates that at least in part, the deleterious effects of A β in AD are caused by its interaction with neuronal apoE and the subsequent effects on cellular apoE pools. This study also demonstrates that perivascular astrocytes representing the glia limitans are required for the perivascular clearance of apoE and, in so doing, for that of apoE-linked A β as well, suggesting that these cells are critically involved in the pathogenesis of AD.

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