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Apolipoprotein E promotes astrocyte colocalization and degradation of deposited amyloid-β peptides

Milla Koistinaho[1,4], Suizhen Lin[1,4], Xin Wu[1], Michail Esterman[2], Deanna Koger[1], Jeffrey Hanson[2], Richard Higgs[3], Feng Liu[1], Seema Malkani[1], Kelly R Bales[1] & Steven M Paul[1]

We have previously shown that apolipoprotein E (Apoe) promotes the formation of amyloid in brain and that astrocyte-specific expression of *APOE* markedly affects the deposition of amyloid-β peptides (Aβ) in a mouse model of Alzheimer disease. Given the capacity of astrocytes to degrade Aβ, we investigated the potential role of Apoe in this astrocyte-mediated degradation. In contrast to cultured adult wild-type mouse astrocytes, adult *Apoe*$^{-/-}$ astrocytes do not degrade Aβ present in Aβ plaque–bearing brain sections *in vitro*. Coincubation with antibodies to either Apoe or Aβ, or with RAP, an antagonist of the low-density lipoprotein receptor family, effectively blocks Aβ degradation by astrocytes. Phase-contrast and confocal microscopy show that *Apoe*$^{-/-}$ astrocytes do not respond to or internalize Aβ deposits to the same extent as do wild-type astrocytes. Thus, Apoe seems to be important in the degradation and clearance of deposited Aβ species by astrocytes, a process that may be impaired in Alzheimer disease.

Alzheimer disease (AD) is characterized by an age-dependent deposition of Aβ peptides, the formation of neurofibrillary tangles, and neurodegeneration in selective regions of the brain. The presence of large numbers of activated glial cells in close proximity to Aβ-containing plaques is an invariant feature of AD neuropathology, although the relationship between glial activation and Aβ deposition remains unclear. Apoe is an abundant 34-kDa glycoprotein that is synthesized and secreted mainly by astrocytes and microglia in the central nervous system (CNS). It is well established that *APOE*, and specifically the *ε4* allele of *APOE*, is a major genetic risk factor for the more common, late-onset form of AD[1,2]. *APOE* genotype also seems to be a determinant of brain Aβ burden in individuals affected with AD[3,4].

Our previous studies have shown that mouse *Apoe* promotes the formation of thioflavine-S–positive amyloid deposits in a manner dependent on the *Apoe* gene dose in a transgenic mouse model of AD[5–7]. By contrast, astrocytic expression of human *APOE* isoforms markedly suppresses deposition of Aβ in this same model[7,8]. In addition, neither mouse *Apoe* nor human *APOE* expression has any significant impact on synthesis of brain Aβ in these transgenic mouse models of AD[5,6], indicating that *APOE* may somehow affect the clearance of Aβ from brain parenchyma.

Microglia are generally recognized as the principal immune effector and phagocytic cell type in the CNS and, indeed, specific activation of microglia with transforming growth factor β1 (TGF-β1)[9] or opsonization of Aβ deposits with antibodies to Aβ[10,11] triggers microglial phagocytosis of Aβ from brain tissue *in vitro* and *in vivo*. Several studies have shown that microglia can phagocytose Aβ, but their effectiveness in degrading amyloid fibrils is limited[12,13]. Ultrastructural studies of postmortem brains from individuals affected with AD also suggest that microglia may even contribute to the formation of amyloid fibrils[14–16]. In addition, activated microglia have been postulated to be involved in neurodegeneration and to exacerbate the inflammation commonly observed in AD brain[17–19].

Astrocytes greatly outnumber microglial cells in the CNS and have vital roles in neuronal development, in maintaining functional synapses, and in CNS repair after injury. Astrocytes also show highly plastic phenotypes, migratory activity, phagocytic and proteolytic capacities[20–24]. Evidence suggests that Aβ peptides are internalized *in vivo* preferentially by astrocytes, and not microglia[25,26], and ultrastructural analysis of AD brain shows that the hypertrophic processes of astrocytes can degrade Aβ-containing plaques[27], indicating that astrocytes may enhance the degradation and clearance of Aβ. Adult (but not neonatal) mouse astrocytes have been also shown to effectively degrade Aβ deposits in brain sections obtained from a mouse model of AD *in vitro*[28].

Given the capacity of astrocytes to degrade Aβ and the fact that astrocyte-specific expression of *APOE* markedly affects Aβ deposition *in vivo*[7,8], we investigated the possible role of Apoe in astrocyte-mediated Aβ degradation. Here we report that Apoe is essential for astrocytes to associate with, respond to, internalize and degrade Aβ deposits in brain sections *in vitro*. Thus, Apoe may enhance brain Aβ clearance by facilitating the ability of astrocytes to find, internalize and degrade deposits of Aβ. Deficits in *APOE*-mediated astrocytic clearance of Aβ may therefore contribute to the pathogenesis of AD.

[1]Neuroscience Discovery Research, [2]Discovery Information Technology, and [3]Genomics Informatics-Statistics, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285, USA. [4]These authors contributed equally to this work. Correspondence should be addressed to S.M.P. (Paul_Steven_M@Lilly.com).

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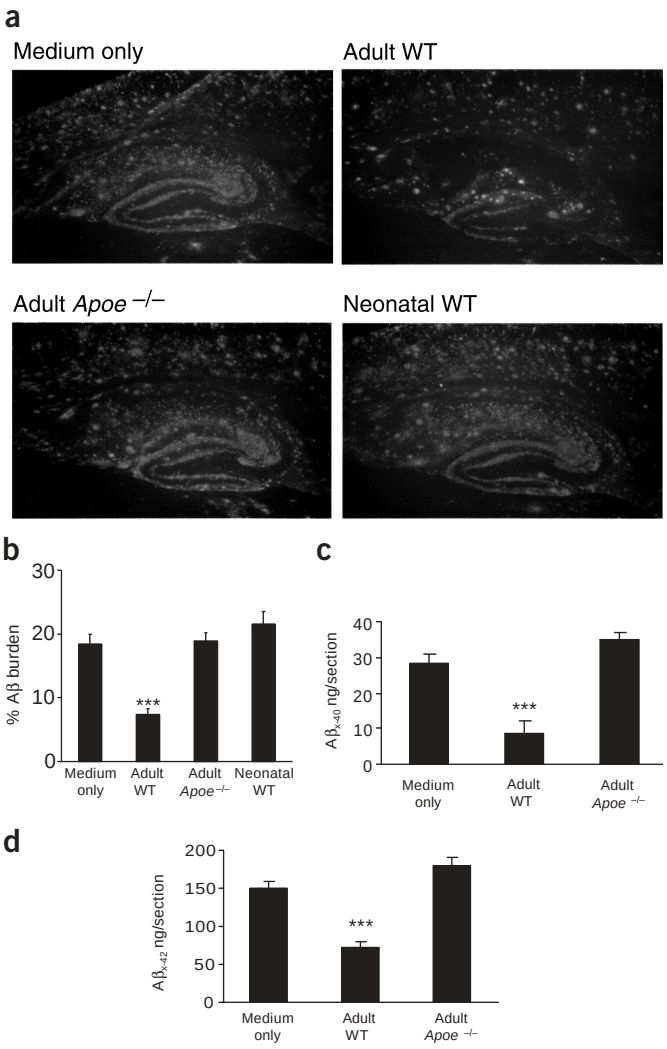

Figure 1 Apoe is essential for the degradation of Aβ by adult astrocytes. (**a**) Representative photomicrograph showing a reduction in Aβ in mouse brain sections after incubation with adult wild-type (WT) astrocytes, as compared with sections incubated with medium only or adult *Apoe*$^{-/-}$ or neonatal wild-type astrocytes. Aβ was detected by IHC with the monoclonal antibody 3D6. (**b**) Aβ burden (percentage area occupied by 3D6 immunoreactivity) of a representative experiment ($n \geq 4$), showing a significant decrease in hippocampal Aβ burden after incubation with adult wild-type astrocytes but not after incubation with adult *Apoe*$^{-/-}$ or neonatal astrocytes, or with medium only ($n = 8$ sections; $^{***}P = 0.001$ versus medium only, or *Apoe*$^{-/-}$ or neonatal astrocytes). (**c**,**d**) Relative amounts of both $A\beta_{x\text{-}40}$ and $A\beta_{x\text{-}42}$ are significantly decreased after incubation of sections with adult wild-type astrocytes but remain unchanged after incubation with *Apoe*$^{-/-}$ astrocytes, as determined by ELISA ($n = 6$; $^{***}P = 0.001$ versus medium only or *Apoe*$^{-/-}$ astrocytes).

RESULTS

Apoe-deficient astrocytes do not degrade Aβ

To study the degradation of human Aβ by astrocytes, we used an *in vitro* assay in which primary mouse astrocytes are cultured on top of unfixed cryostat-prepared sections from old PDAPP transgenic mice, which overexpress human amyloid precursor protein and have abundant deposits of Aβ primarily in the form of nonfibrillar plaques. After incubation with exogenous astrocytes for 24 h or longer, the sections were initially processed for quantifying Aβ burden by immunohistochemistry (IHC) using the 3D6 antibody, which detects intact N-terminal Aβ peptides.

As reported previously[28], incubation of PDAPP mouse brain sections for 24 h with adult wild-type mouse astrocytes reduced Aβ burden in hippocampi by up to 50% ($P = 0.001$; **Fig. 1a**,**b**). By contrast, when adjacent PDAPP brain sections were incubated with adult mouse astrocytes prepared from *Apoe* knockout (*Apoe*$^{-/-}$) mice, there was little to no reduction in Aβ burden (**Fig. 1a**,**b**). As shown previously[28], neonatal wild-type astrocytes did not degrade Aβ under identical experimental conditions (**Fig. 1a**,**b**). Adult wild-type astrocytes were also less effective at degrading Aβ in tissue sections from PDAPP mouse brain with a low Aβ plaque burden (data not shown).

We next used a sandwich enzyme-linked immunosorbent assay (ELISA) to detect human $A\beta_{x\text{-}40}$ and $A\beta_{x\text{-}42}$ in brain sections after incubation with adult mouse astrocytes. Adult wild-type astrocytes reduced $A\beta_{x\text{-}40}$ and $A\beta_{x\text{-}42}$ levels by 69 and 52%, respectively ($P = 0.001$), whereas *Apoe*$^{-/-}$ astrocytes did not degrade either Aβ species (**Fig. 1c**,**d**). When we determined Aβ burden, as well as $A\beta_{x\text{-}40}$ and $A\beta_{x\text{-}42}$, after 48 h of incubation with adult mouse wild-type or *Apoe*$^{-/-}$ astrocytes, similar results were obtained (data not shown). Thus, *Apoe*$^{-/-}$ astrocytes cannot remove preexisting human Aβ deposits even after longer incubation times.

Because the remaining Aβ deposits in sections incubated with adult wild-type astrocytes seemed to be compact in nature, we stained PDAPP mouse brain sections with thioflavine-S 24 h after incubation with adult wild-type and *Apoe*$^{-/-}$ astrocytes to quantify the number of fibrillar dense-core amyloid deposits. Even though adult wild-type astrocytes effectively cleared diffuse Aβ deposits from PDAPP mouse brain sections, the hippocampal amyloid burden remained unchanged ($P > 0.05$; **Supplementary Fig. 1** online).

Astrocyte viability is unaffected by the absence of Apoe

Could variations in cell viability account for the marked difference between adult wild-type and *Apoe*$^{-/-}$ astrocytes in degrading Aβ? Using fluorescein diacetate (FDA) and propidium iodide (PI) vital staining (Methods), we found no difference in astrocyte viability (wild type versus *Apoe*$^{-/-}$) either before or after exposure to PDAPP mouse brain sections.

To rule out more subtle changes in cell viability or metabolism, we used [^{35}S]methionine and [^{3}H]thymidine incorporation to measure protein and DNA synthesis, respectively. No differences in protein or DNA synthesis between adult wild-type and *Apoe*$^{-/-}$ astrocytes were observed either before or after exposure to PDAPP mouse brain sections (**Supplementary Fig. 1** online).

RAP and antibodies to Apoe and Aβ block Aβ degradation

We assessed whether incubating astrocytes with an antibody to Apoe would inhibit the degradation of human Aβ by adult wild-type astrocytes. In the presence of normal assay medium, an irrelevant monoclonal IgG (50 μg/ml) or normal rabbit sera (NRS; 50 μg/ml), adult wild-type astrocytes effectively reduced hippocampal Aβ burden as expected (**Fig. 2a**,**b**); however, the presence of antisera to murine Apoe (50 μg/ml) in adjacent PDAPP mouse brain sections completely blocked the degradation of Aβ by adult wild-type astrocytes (**Fig. 2a–c**).

To address whether Apoe associated with the Aβ deposits themselves, rather than the expression or secretion of Apoe from astrocytes, contributes to the astrocyte-mediated degradation of Aβ, we preincubated the tissue sections or cells with antisera to Apoe (or NRS as a control) for 2 or 24 h, removed the antisera, and then exposed the sections to adult wild-type astrocytes. Preincubating the tissue sections or the cells with antisera did not block the ability of wild-type astrocytes to degrade Aβ, in marked contrast to the almost

complete blockade observed when the astrocytes were coincubated with antisera (**Supplementary Fig. 2** online).

Antibodies to Aβ have been reported to stimulate microglial phagocytosis of Aβ *in vitro*, possibly through an Fc receptor–mediated mechanism (ref. 10, but see ref. 11). Because some astrocytes may express Fc receptors[29], we studied the effects of various antibodies to Aβ on astrocytic clearance of Aβ deposits. We first preincubated PDAPP mouse brain sections with a polyclonal antibody to Aβ (Aβ pan), a monoclonal antibody to Aβ (10D5) or irrelevant IgGs (antibodies to PSD95 or to tau phosphorylated on Ser202; 5 μg/ml each) for 24 h, and then added adult wild-type astrocytes for 24 h before measuring the concentrations of human $Aβ_{x-40}$ and $Aβ_{x-42}$ by ELISA. Adult wild-type astrocytes degraded Aβ from brain sections in the presence of either irrelevant IgG (**Fig. 2c**,**d**), but not when the sections were preincubated with either of the antibodies to Aβ (**Fig. 2 c**,**d** and data not shown).

We next assessed whether treatment with RAP, the 39-kDa receptor–associated protein and low-density lipoprotein (LDL) receptor family antagonist[30] would block the adult wild-type astrocyte–mediated degradation of Aβ. Preincubation (2 h) or coincubation of adult wild-type astrocytes with RAP (50 μg/ml) significantly blocked Aβ degradation by wild-type astrocytes during incubation for a total of 24 h with PDAPP mouse brain sections (**Fig. 2e**).

Astrocyte association and response to Aβ is facilitated by Apoe

The presence of numerous astrocytes in close proximity to Aβ deposits in AD brain suggests that Aβ and other plaque-associated molecules may be chemoattractant factors for astrocytes[25,28]. When we incubated exogenous astrocytes with brain sections from a high-plaque–bearing PDAPP mouse brain (hippocampal Aβ burden ≥ 25%), we observed that adult wild-type astrocytes formed rather large multicellular aggregates with thick extended processes that, in many cases, appeared to form networks between other multicellular aggregates in Aβ-rich brain areas such as the hippocampus (**Fig. 3**). These very large multicellular aggregates were not observed (the aggregates were much smaller with few, if any, extended processes) when *Apoe*$^{-/-}$ astrocytes were incubated with adjacent brain sections (**Fig. 3**) or when adult wild-type astrocytes were incubated with wild-type mouse brain sections (**Supplementary Fig. 3** online). In the presence of antibody to Apoe, adult wild-type astrocytes showed very limited aggregation and did not develop the extended processes observed in wild-type astrocytes in the absence of antibody (**Fig. 3**).

We also found significantly fewer *Apoe*$^{-/-}$ astrocytes over the hippocampus, and quantification of mean aggregate areas in the hippocampal region verified significantly smaller aggregates ($P = 0.001$; **Fig. 4a**), when PDAPP mouse brain sections were incubated with *Apoe*$^{-/-}$ astrocytes. Neither adult wild-type nor *Apoe*$^{-/-}$ astrocytes

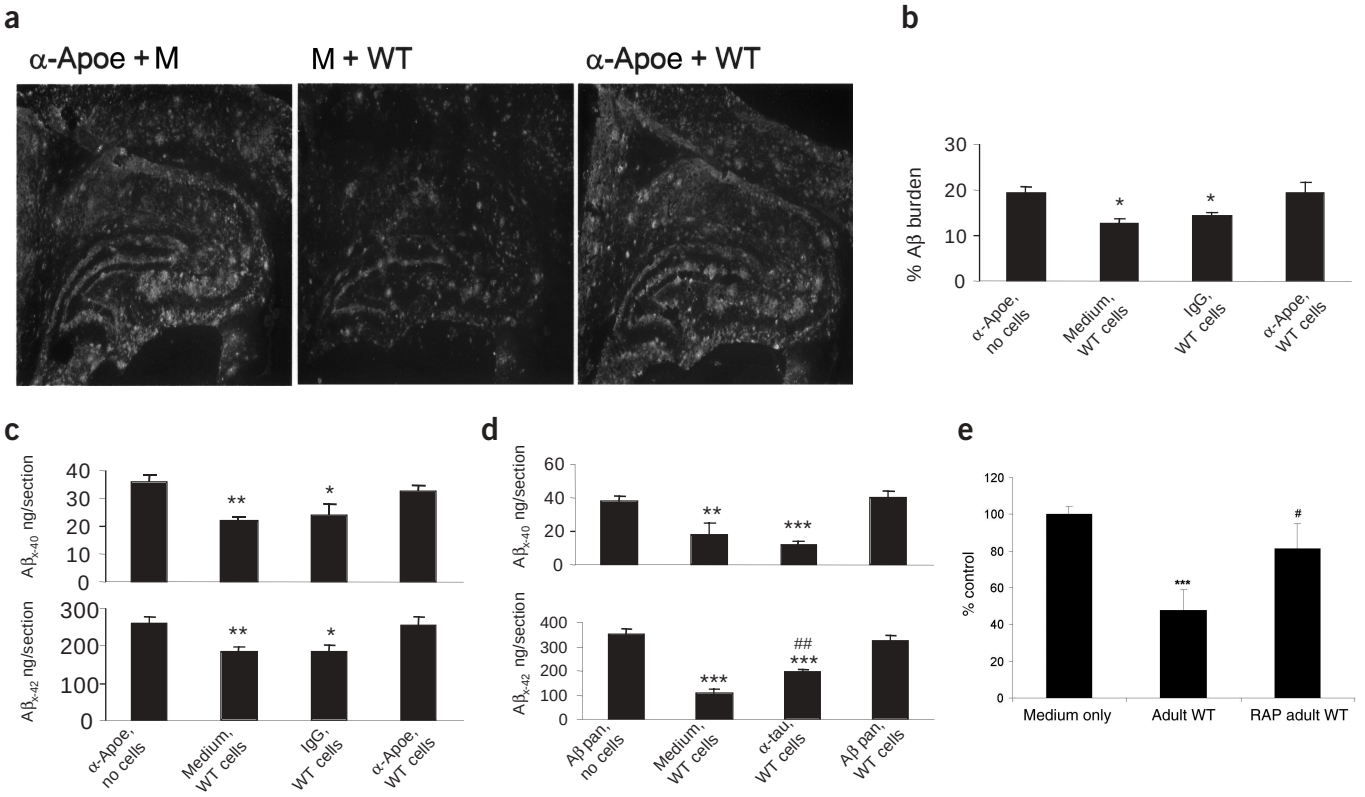

Figure 2 Antibodies to Apoe and Aβ block degradation of Aβ. (**a**) Representative photomicrograph showing IHC detection of Aβ after incubation of brain sections with antisera to Apoe plus medium alone (α-Apoe + M), adult wild-type astrocytes (M + WT) or antisera to Apoe plus adult wild-type astrocytes (α-Apoe + WT). (**b**,**c**), Aβ burden measured by IHC and Aβ levels measured by ELISA show a decrease in Aβ after incubation with adult wild-type astrocytes in the presence (IgG, WT cells) or absence (medium, WT cells) of an irrelevant IgG or NRS. No significant decease in Aβ burden was seen after incubation with antisera to Apoe either alone (α-Apoe, no cells) or plus adult wild-type astrocytes (α-Apoe, WT cells; $n = 6$–8 sections; $^*P < 0.05$). (**d**) Preincubation of brain sections with a polyclonal antibody to Aβ (Aβ pan, no cells) or an antibody to tau plus adult wild-type astrocytes (α-tau, WT cells) does not block degradation of $Aβ_{x-40}$ and $Aβ_{x-42}$ by adult wild-type astrocytes, whereas preincubation with an antibody to Aβ plus wild-type astrocytes (Aβ pan, WT cells) does ($n = 5$–6 sections; $^{***}P = 0.001$, $^{**}P = 0.01$, medium plus astrocytes versus Aβ pan either alone or plus astrocytes; $\#\#P = 0.01$ versus Aβ pan plus astrocytes). (**e**) Coincubation of brain sections with RAP (50 μg/ml) inhibits the ability of adult wild-type astrocytes to degrade Aβ ($n = 8$ sections; $^{***}P = 0.001$, astrocytes versus medium only or RAP plus astrocytes; $\#P = 0.05$, RAP plus astrocytes versus medium only).

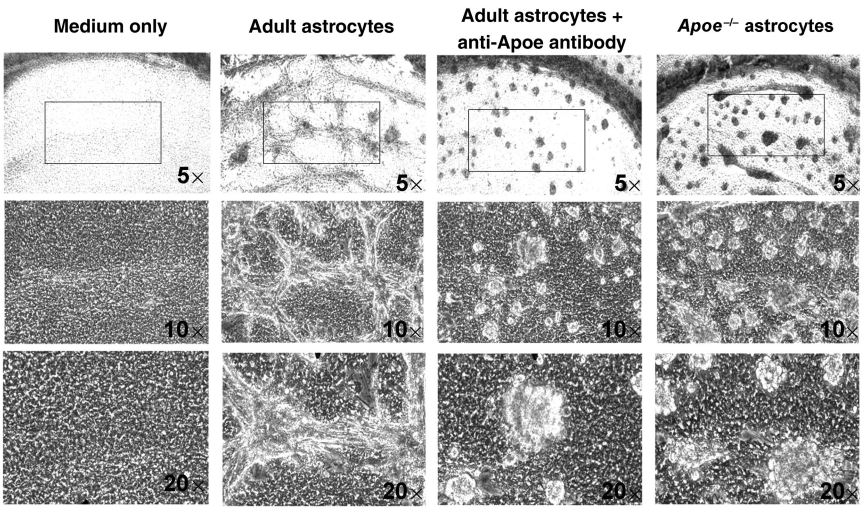

Figure 3 Adult wild-type astrocytes aggregate and form extended processes. Representative phase-contrast photomicrographs of PDAPP mouse brain sections after incubation with medium only, adult wild-type astrocytes, adult wild-type astrocytes plus antibody to Apoe (Adult astrocytes + anti-Apoe antibody) or adult *Apoe*$^{-/-}$ astrocytes. Note the larger size of the multicellular aggregates and the thick extended processes that appear to form interconnecting networks when adult wild-type astrocytes are exposed to PDAPP mouse brain sections for 24 h. Smaller aggregates with relatively fewer processes were observed when adult wild-type astrocytes were exposed to serial sections from the same brain tissue in the presence of antibody to Apoe. Note that similar results were observed with *Apoe*$^{-/-}$ astrocytes.

formed large aggregates in brain areas devoid of Aβ deposits such as the cerebellum (**Fig. 4a**), suggesting that Apoe might be involved in the recruitment and/or aggregation of astrocytes in response to Aβ.

The good spatial separation of Aβ deposits in a low-plaque–bearing PDAPP mouse brain (Aβ burden ≈ 4%) enabled us to determine the association of exogenously added astrocytes with these Aβ deposits after 24 h of incubation. We counted the number of exogenous astrocytes that surrounded or touched 10–12 randomly chosen plaques per hippocampus. For 60 randomly identified plaques analyzed per group, we observed that at least six times more adult wild-type astrocytes than *Apoe*$^{-/-}$ astrocytes, on average, were associated with each Aβ deposit ($P = 0.001$; **Fig. 4b**). Incubation of adult wild-type astrocytes and PDAPP mouse brain sections with antibodies to either Apoe or Aβ reduced the association of astrocytes with Aβ deposits, as compared with an irrelevant IgG, although not to the same extent as the reduction observed with *Apoe*$^{-/-}$ astrocytes ($P = 0.001$, **Fig. 4c**).

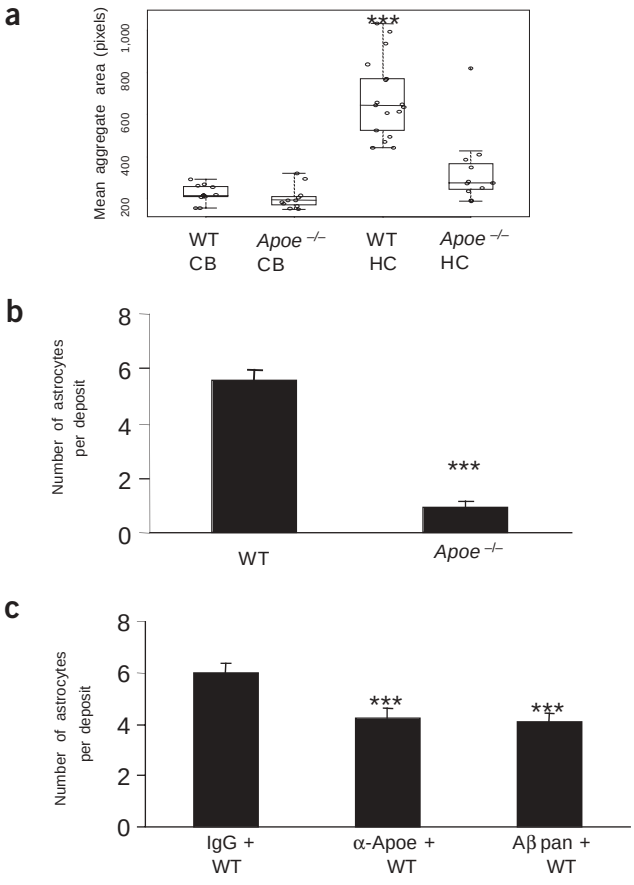

Figure 4 Apoe is important for astrocytes to associate with Aβ. (**a**) Adult wild-type (WT) astrocytes form larger multicellular aggregates than do *Apoe*$^{-/-}$ astrocytes. Mean aggregate areas in the cerebellum (CB) or hippocampus (HC) of PDAPP brain sections were determined after incubation with adult wild-type or *Apoe*$^{-/-}$ astrocytes for 24 h (***$P = 0.0001$). (**b**) After 24 h of incubation with low plaque–bearing mouse brain sections, adult *Apoe*$^{-/-}$ astrocytes associate with Aβ deposits significantly less than do wild-type (WT) astrocytes (***$P = 0.001$, $n = 50$–60 plaques). (**c**) Antibodies to Apoe and Aβ inhibit the association of adult wild-type astrocytes with Aβ plaques (***$P = 0.001$).

Astrocyte aggregates are associated with Aβ degradation

To visualize better the association of exogenously added astrocytes with Aβ deposits, we infected adult wild-type and *Apoe*$^{-/-}$ astrocytes with a lentiviral vector encoding green fluorescent protein (GFP) before seeding them onto high-plaque–bearing PDAPP mouse brain sections for 24 h. Infection of adult wild-type astrocytes with the lenti-GFP construct did not alter their ability to degrade Aβ (data not shown). Notably, after incubation, Aβ immunoreactivity underneath the multicellular aggregates formed by adult wild-type astrocytes had almost disappeared, forming cavities or craters within the deposits themselves (**Fig. 5a**). By contrast, even though some *Apoe*$^{-/-}$ astrocytes were occasionally found in high-plaque brain areas, the adjacent Aβ surfaces seemed to be intact and no craters were observed (**Fig. 5b**).

It is important to note that the craters observed in the Aβ deposits were not observed in serial sections incubated with *Apoe*$^{-/-}$ astrocytes, suggesting that they formed after exposure to the adult wild-type astrocytes and not before. Similarly, adult wild-type astrocytes preincubated with high-plaque–bearing PDAPP mouse brain sections in the presence of antibodies to Apoe or Aβ did not form large multicellular aggregates above the plaques and did not degrade Aβ underneath them (**Fig. 5c**).

Apoe-dependent internalization of Aβ by astrocytes

We used confocal microscopy to visualize better the surfaces of exogenous astrocytes and Aβ in PDAPP mouse brain sections double-

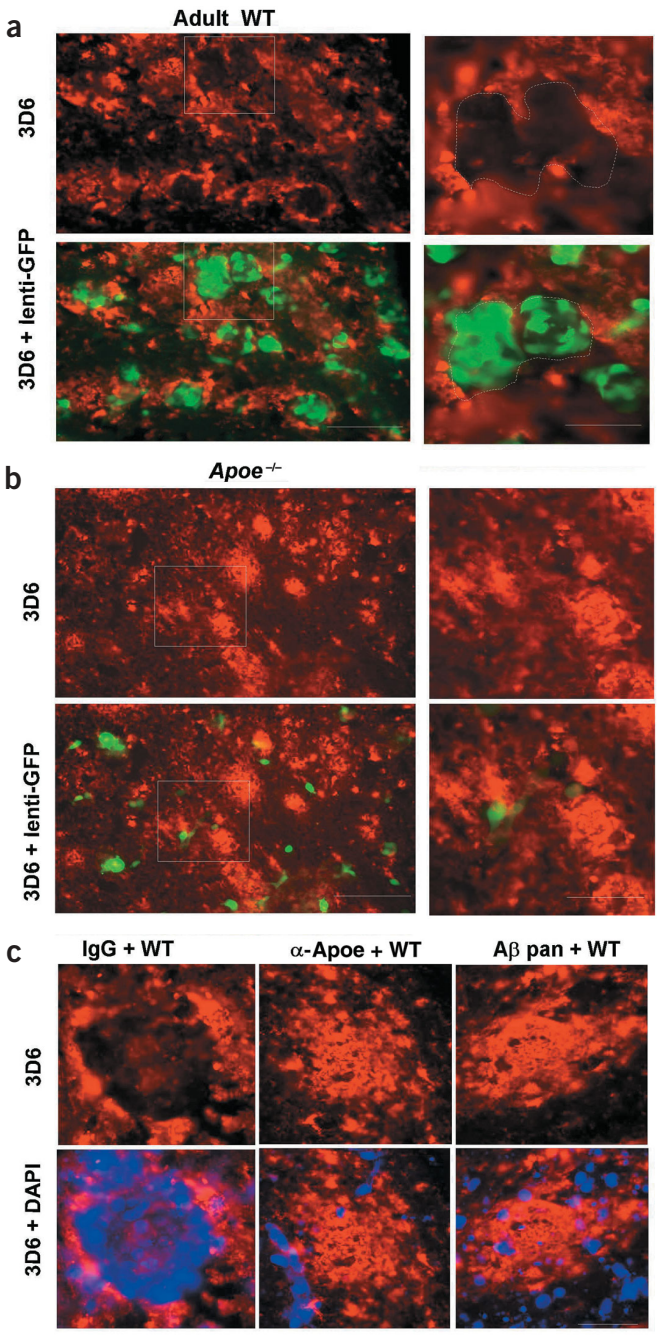

Figure 5 Multicellular aggregates of astrocytes are associated with loss of Aβ. (**a**,**b**) Representative images of hippocampal sections immunostained with 3D6 (top) and merged with images from the same field using a green filter to visualize GFP-expressing adult wild-type (**a**) and *Apoe*$^{-/-}$ (**b**) astrocytes (bottom). (**a**) Adult wild-type astrocytes remove Aβ from surfaces underneath. The white boxes and dotted lines show areas of cavitation (Aβ loss) in the high-power photomicrographs. (**b**) The few small aggregates of astrocytes are not associated with loss of Aβ deposition. Scale bars, 200 μm (lower power); 100 μm (higher power). (**c**) In the presence of control antibody, adult wild-type astrocytes, visualized by blue DAPI staining of nuclei (bottom), remove Aβ-immunoreactive material from PDAPP mouse brain sections (top). Incubation of adult wild-type astrocytes with antibodies to Apoe or Aβ blocks the formation of multicellular aggregates and the removal of Aβ-immunoreactive material.

stained with anti–glial fibrillary acidic protein (GFAP) and 3D6 antibodies. We collected serial images from brain sections incubated with exogenous astrocytes starting at a level 5.2-μm deep in the section and proceeding upwards at 0.4-μm intervals. Reconstructed surface images at increasing incubation times showed that at an early time point the astrocytes from each group showed similar morphology (**Fig. 6a**), but their distribution in the hippocampus differed between the groups.

Similar to the association analysis using light microscopy (**Fig. 4a**), adult wild-type astrocytes preferred Aβ-rich areas, whereas the few *Apoe*$^{-/-}$ astrocytes found in the hippocampus were mainly localized to areas of low Aβ burden. The morphology of adult wild-type, but not *Apoe*$^{-/-}$ or neonatal astrocytes, had also changed markedly at 24 h. As observed with phase-contrast microscopy, adult wild-type astrocytes showed a hypertrophic and more flattened phenotype with highly extended processes that often engulfed the Aβ deposits (**Fig. 6a**,**b**). By contrast, neonatal and *Apoe*$^{-/-}$ astrocytes had a more globular shape at this time point.

We quantified the area of Aβ in direct contact with adult wild-type astrocytes by using confocal images collected from hippocampal regions of PDAPP brain sections at 24 h and found a 75-fold and 226-fold increase in area as compared with *Apoe*$^{-/-}$ and neonatal wild-type astrocytes, respectively (P = 0.001; **Fig. 6c**). Despite a change in morphological phenotype from a globular to more flat and ramified shape, *Apoe*$^{-/-}$ astrocytes had not significantly engulfed Aβ after 48 h of incubation (**Fig. 6c**).

High-resolution confocal images showed that adult wild-type astrocytes effectively internalized Aβ (**Fig. 6d**), whereas *Apoe*$^{-/-}$ astrocytes did not. To quantify this phenomenon, we determined the percentage of exogenous GFAP immunoreactivity (green) that colocalized with Aβ (red) at 24 h. Quantification of colocalization coefficients as a measure of internalization showed that 38% of the volume of adult wild-type astrocytes was occupied by Aβ (**Fig. 6e**). The corresponding value for adult *Apoe*$^{-/-}$ astrocytes was 0.5% or less.

DISCUSSION

We have shown that adult mouse astrocytes associate with, morphologically respond to, internalize and degrade deposited Aβ peptides from brain parenchyma *in vitro*, and that all of these features are substantially or completely reduced in the absence of Apoe expression. Our data confirm and extend findings showing that adult mouse astrocytes have the capacity to degrade deposited human Aβ peptides effectively *in vitro*[28]. By contrast, cultured neonatal astrocytes were ineffective at degrading Aβ under identical assay conditions, suggesting that they may be poor models for studying some diseases of the adult nervous system.

Given the role of *APOE* (expressed only by astrocytes via an astrocyte-specific GFAP promoter) in altering brain Aβ burden in PDAPP mice *in vivo*[7,8], and the fact that astrocytes are the most abundant source of Apoe in brain, we examined whether Apoe expression is important in mediating Aβ degradation by astrocytes. In comparison to those prepared from wild-type mice, astrocytes prepared from *Apoe*$^{-/-}$ mice were unable to degrade Aβ deposits present in PDAPP mouse brain sections. In addition, preincubation of PDAPP brain sections and wild-type astrocytes with an antibody to either Apoe or Aβ markedly reduced degradation of Aβ, suggesting that a physical interaction of Apoe and Aβ may be required for Aβ degradation by astrocytes.

Apoe can form stable complexes with Aβ, and CNS lipoproteins containing Apoe may clear brain Aβ via lipoprotein receptors. In one study[31], Apoe seemed to form complexes more readily with soluble Aβ than with aggregated Aβ, and these complexes were taken up by synap-

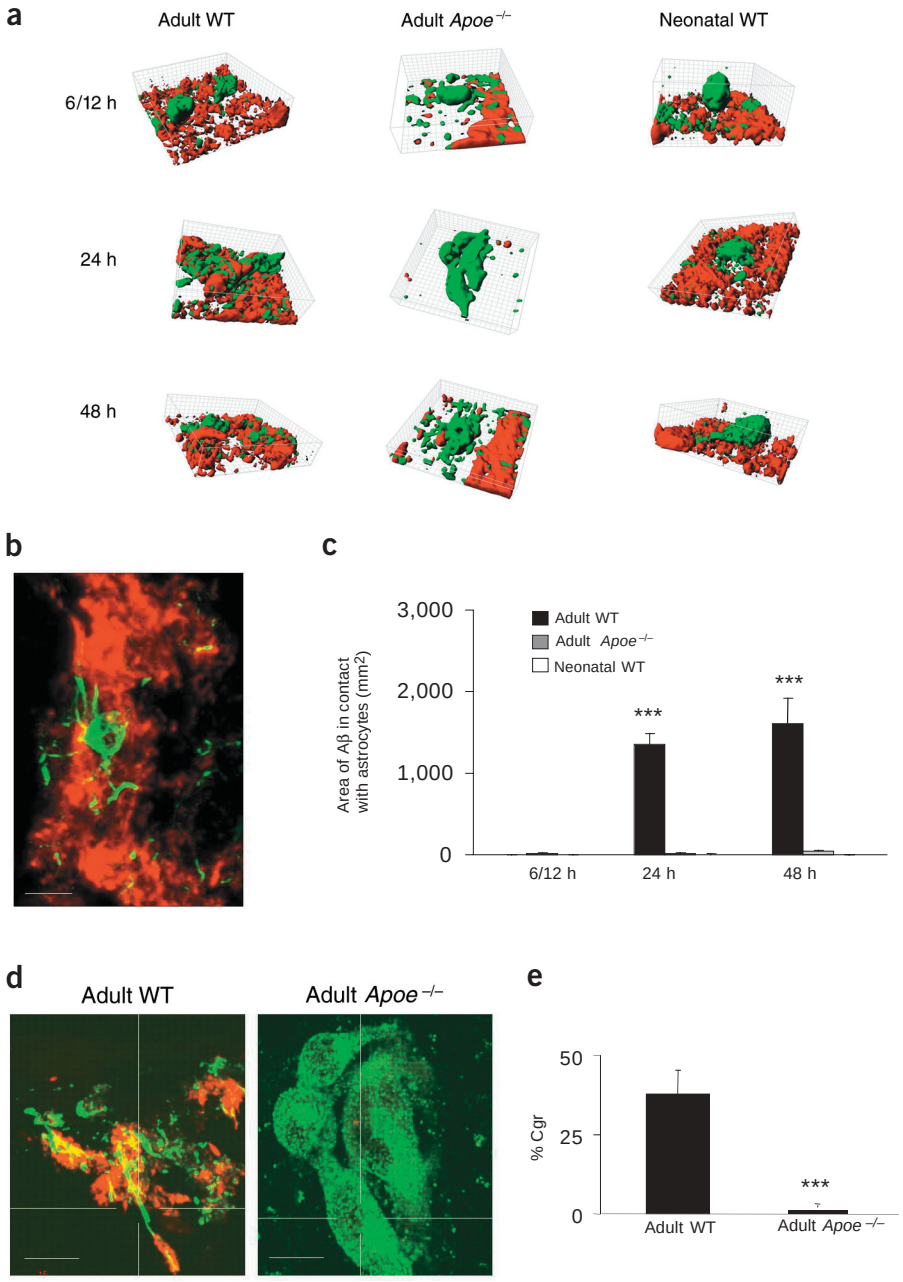

Figure 6 Adult wild-type astrocytes associate with and internalize human Aβ. (**a**) Reconstructed surface images from high-plaque brain sections incubated with exogenous adult wild-type (left), adult *Apoe*$^{-/-}$ (middle) or neonatal wild-type (right) astrocytes and immunostained for GFAP (green) and Aβ (red) show time-dependent changes in the morphology of adult wild-type astrocytes. (**b**) A maximum intensity projection image captured above the tissue plane shows an astrocyte that has 'lifted' Aβ by engulfing it within its processes. Scale bar, 15 μm. (**c**) Quantification of the area of Aβ in contact with GFAP-positive astrocytes is increased for adult wild-type astrocytes between 12 and 24 h (n = 4–21 astrocytes per group per time point; P = 0.001, versus other groups). (**d**) Confocal images collected 10 μm above the surface of the brain section show that Aβ is localized in (yellow) and around (red) exogenous adult wild-type astrocytes (green) at 24 h. Scale bars, 8 μm. (**e**) Quantification of the percentage of green (GFAP) pixels containing a red (Aβ) component (% Cgr) reveals a roughly 80-fold difference between adult wild-type and *Apoe*$^{-/-}$ astrocytes (n = 3–4 images with 3–8 cells on each; ***P = 0.001).

tic terminals via the LDL receptor–related protein. Similar results have been reported for the internalization of Aβ by human and canine cerebrovascular smooth muscle cells[32]. In our hands amyloid itself was not readily degraded by adult wild-type astrocytes; however, it is clear that diffuse plaques containing aggregated Aβ were readily cleared.

In addition, RAP, an LDL receptor family antagonist[30] was also effective in blocking astrocyte-mediated Aβ degradation. This suggests that either the LDL receptor itself or the LDL receptor–related protein, which are both expressed on astrocytes, may be responsible for mediating uptake and degradation of the Apoe–Aβ complex. We also examined whether the relative inability of adult *Apoe*$^{-/-}$ astrocytes to degrade Aβ was due to reduced viability or an inability to divide and/or proliferate in primary culture. The data suggest that the inability of *Apoe*$^{-/-}$ astrocytes to degrade Aβ is not due to reduced viability or to a generalized impairment of either DNA or protein synthesis.

After being exposed to PDAPP mouse brain sections, adult mouse wild-type astrocytes formed large multicellular aggregates with thick extended processes, especially in brain regions with high Aβ burden. These multicellular aggregates of astrocytes with extended processes were themselves associated with areas of reduced Aβ deposition. In fact, the multicellular aggregates seemed to form craters in the Aβ deposits that were not observed in contiguous serial brain sections exposed to *Apoe*$^{-/-}$ or neonatal astrocytes, suggesting that they were formed by the adult wild-type astrocytes themselves. Quantification of the number and size of these multicellular aggregates identified large differences between wild-type and *Apoe*$^{-/-}$ astrocytes in high-plaque–bearing brain regions.

Treatment with antibody to Apoe blocked the ability of wild-type astrocytes to degrade Aβ and markedly inhibited formation of the multicellular aggregates and the thick extended processes that were observed in the absence of antibody. Taken together, these data suggest that the Apoe that is expressed and secreted by astrocytes is essential for the astrocytic response to Aβ deposits. Finally, although the exact mechanism underlying the Apoe-dependent internalization of Aβ by adult wild-type astrocytes has not been fully elaborated, our data favor a receptor-mediated process that probably involves a member of the LDL receptor family.

Using confocal microscopy we followed over time the association of adult wild-type and *Apoe*$^{-/-}$ astrocytes with Aβ deposits. *Apoe*$^{-/-}$ astrocytes, in contrast to adult wild-type astrocytes, had a more globular shape with little aggregation and fewer processes. High-resolution images showed that adult wild-type astrocytes effectively internalized Aβ present in plaques, whereas *Apoe*$^{-/-}$ astrocytes did not. These data suggest that Apoe facilitates the internalization and degradation of Aβ by astrocytes.

Our experimental model, in which exogenous astrocytes and brain sections are studied *in vitro*, does not address the issue of whether a similar process occurs *in vivo*; however, it has been shown that astrocytes and not microglia are the principal Aβ-accumulating cells in the rat brain[26]. In addition, ultrastructural studies of AD postmortem brain have consistently shown the presence of Aβ-containing astrocytes[33,34], especially in association with diffuse plaques[27,34]. Those studies also suggest that astrocytes (in contrast to microglia) are associated with plaque degradation and clearance[27,34]. Neither do our findings address whether variations in *APOE* genotype translate to differences in astrocyte-mediated Aβ degradation. Further work will be required to address both of these issues. We propose, however, that intrinsic alterations in the ability of astrocytes to degrade Aβ, due to either aging or *APOE* genotype (or both), could be involved in the pathogenesis of AD and thus might offer a completely different approach to therapeutic intervention.

METHODS

Astrocyte cultures. Adult mouse astrocytes were cultured as described[35] with the following modifications. Hippocampi and cortices were obtained from 20 C57BL/6 (Taconic) or C57BL/6 *Apoe*$^{-/-}$ (Taconic) mice, aged 7 weeks, in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution. The suspension was treated with 0.25% trypsin and 1 mM EDTA for 30 min at 37 °C and then triturated with 20 U/ml DNase I (Invitrogen). Cells were washed with growth medium consisting of DMEM/F12 (3:1), 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Gibco) and centrifuged at 1,500 rpm for 5 min. Dissociated cells were filtered through a 100-μm cell strainer (Becton Dickinson), treated with Percoll (ICN) and resuspended in growth medium with G5 supplement (Invitrogen) to establish primary cultures.

We prepared neonatal astrocyte cultures as described[36] from mice aged 1 d. All cultures used in these experiments were determined to comprise more than 99% astrocytes by IHC. Astrocyte viability was measured by counting the ratios of living to dead cells in FDA and PI double-stained samples. In some experiments, primary astrocytes were infected with a lentiviral vector expressing GFP driven by the human cytomegalovirus promoter[37] (a gift of I. Verma and R. Marr, Salk Institute, La Jolla, CA).

***In vitro* Aβ degradation assay and IHC.** All mouse experiments were approved by the Eli Lilly and Company Institutional Animal Care and Use Committee. Heterozygous C57BL/6 APP(V717F)$^{+/-}$ (PDAPP) mice (aged 22–26 months) were perfused with saline and the brains were frozen on dry ice. Sagittal sections (10 μm) were cut on a CM 3050-S cryostat (Leica), mounted on poly-L-lysine–coated coverslips, transferred to two-well chamber slides and used immediately or stored at −80 °C until use. Astrocytes were seeded in the chamber at a density of 5×10^5 cells in 1 ml of assay medium (DMEM/F12, G5 supplement, 0.2% bovine serum albumin (BSA), penicillin and streptomycin) and the cultures were maintained for 24 h or longer at 37 °C (ref. 28).

For antibody experiments, assay medium or antibody at the specified concentration was incubated with the sections for 2–24 h before the addition of the cells. We used the following: rabbit anti–mouse Apoe (50 μg/ml; Biodesign), rabbit anti–mouse IgG (5 or 50 μg/ml; Chemicon), Aβ pan (5 μg/ml; Biosource), 10D5 monoclonal antibody to Aβ (5 μg/ml), anti–mouse IgG (5 μg/ml; Harlan), rabbit anti–PSD95 CT (5 μg/ml; Zymed), rabbit antibody to tau phosphorylated on Ser202 (5 μg/ml; Biosource), vehicle used in antibody preparations, or RAP[30] (50 μg/ml; a gift of G. Bu, Washington University, St. Louis, MI). After incubation, the sections were fixed with 4% paraformaldehyde in PBS, stained with 0.1% thioflavine-S (Sigma) or permeabilized and blocked with 0.05% BSA in PBS-T. IHC was done with 3D6 (1.7 μg/ml) and rhodamine-conjugated secondary (Chemicon) antibodies.

We acquired phase-contrast images on a DM IRB microscope (Leica Microsystems) equipped with an attached SPOT camera (Diagnostic Instruments). The percentage area of the hippocampus occupied by fluorescent Aβ or thioflavine-S staining was measured in at least 6–8 sections per treatment by an Image-Pro Plus (Media Cybernetics) macro written in Image-Pro scripting language and run under Image-Pro Plus 4.5.0.29. For confocal microscopy, sections were immunostained with 3D6 and anti-GFAP antibodies (diluted 1:1,000). After being washed three times with PBS-T, the sections were incubated with rhodamine-conjugated goat anti–mouse IgG (Chemicon) and Alexa Fluor 488–conjugated goat anti–rabbit IgG (Molecular Probes) and then covered with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI)-containing fluorescent mounting media (Vector) and a coverslip.

Confocal microscopy and image analysis. Images were collected on an MRC1024-UV Confocal System (Bio-Rad) equipped with a 40× Plan Apo, numerical aperture 1.3, oil immersion objective on a Diaphot 200 inverted microscope (Nikon). The green, blue and red fluorescent images were collected sequentially with Lasersharp 2000 (Bio-Rad) acquisition software. The confocal image stacks (in PIC format) were reconstructed by Imaris 3.3.2 and Surpass (Bitplane AG). The quantification of the surface contact between astrocytes and Aβ was done by a macro developed in LaserPix (Bio-Rad). For each confocal section, the astrocyte intensity (green signal) was mathematically 'ANDed' (the sum of the intersection of the area occupied by Aβ in the astrocytes) with the Aβ intensity (red signal) to calculate the area of contact. The total area of contact was computed by summing the individual area of contact of all confocal sections:

$$\text{total contact area} = \Sigma A_{i\ (\text{astrocyte})}\ \text{Åø}\ A_{i\ (A\beta)}$$

The amount of Aβ colocalized with astrocytes was determined by the colocalization module in LaserPix, which uses published algorithms[38]. The reported value is the colocalization coefficient Cgr; the fraction of green pixels that have a red component is determined by

$$\text{Cgr} = \frac{\Sigma G_{i\ (\text{coloc})}}{\Sigma G_i}$$

where G_i = green intensity. To detect and differentiate the multicellular aggregates, an Image-Pro Plus (Media Cybernetics) 'color cube' color range was defined and used across all phase-contrast images to detect the area of astrocytes. Any area smaller than 100 pixels was discarded as noise.

DNA and protein synthesis. We evaluated DNA and protein synthesis by measuring [^{3}H]thymidine and [^{35}S]methionine incorporation, respectively (see **Supplementary Fig. 1** online).

Human Aβ ELISA. After incubation, the tissue sections were washed with 0.1 M PBS and Aβ was extracted by homogenization in 200 μl of 5.5 M guanidine-HCl. $A\beta_{x\text{-}40}$ and $A\beta_{x\text{-}42}$ were quantified by a sandwich ELISA[6]. Antibody 266 and antibodies 2G3 ($A\beta_{x\text{-}40}$) and 21F12 ($A\beta_{x\text{-}42}$) were used as the capture and reporter antibodies, respectively. Quantities of Aβ are expressed as $A\beta_{x\text{-}40}$ or $A\beta_{x\text{-}42}$ per tissue section.

Statistical analyses. To compare differences between the experimental groups, a two-tailed *t*-test or one-way analysis of variance (ANOVA) was done by GraphPad Prizm software (GraphPad Software). The mean aggregate areas in the imaged sections were compared by Tukey's HSD test and JMP 4.0.4. software (SAS Institute).

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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