Loss of clusterin shifts amyloid deposition to the cerebrovasculature via disruption of perivascular drainage pathways

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Alzheimer’s disease (AD) is characterized by amyloid-\(\beta\) (A\(\beta\)) peptide deposition in brain parenchyma as plaques and in cerebral blood vessels as cerebral amyloid angiopathy (CAA). CAA deposition leads to several clinical complications, including intracerebral hemorrhage. The underlying molecular mechanisms that regulate plaque and CAA deposition in the vast majority of sporadic AD patients remain unclear. The clusterin (CLU) gene is genetically associated with AD and CLU has been shown to alter aggregation, toxicity, and blood-brain barrier transport of A\(\beta\), suggesting it might play a key role in regulating the balance between A\(\beta\) deposition and clearance in both brain and blood vessels. Here, we investigated the effect of CLU on A\(\beta\) pathology using the amyloid precursor protein/presenilin 1 (APP/PS1) mouse model of AD amyloidosis on a Clu\textsuperscript{+/+} or Clu\textsuperscript{−/−} background. We found a marked decrease in plaque deposition in the brain parenchyma but an equally striking increase in CAA within the cerebrovasculature of APP/PS1;Clu\textsuperscript{−/−} mice. Surprisingly, despite the several-fold increase in CAA levels, APP/PS1;Clu\textsuperscript{−/−} mice had significantly less hemorrhage and inflammation. Mice lacking CLU had impaired clearance of A\(\beta\) in vivo and exogenously added CLU significantly prevented A\(\beta\) binding to isolated vessels ex vivo. These findings suggest that in the absence of CLU, A\(\beta\) clearance shifts to perivascular drainage pathways, resulting in fewer parenchymal plaques but more CAA because of loss of CLU chaperone activity, complicating the potential therapeutic targeting of CLU for AD.

Significance

Deposition of amyloid-\(\beta\) (A\(\beta\)) peptide in the form of parenchymal plaques and A\(\beta\) accumulation in the walls of cerebral vessels as cerebral amyloid angiopathy (CAA) are pathological hallmarks of Alzheimer’s disease (AD). The clusterin (CLU) gene, which confers AD risk, is associated with amyloid deposition. Here we show that loss of CLU promotes cerebrovascular CAA, yet significantly reduces the amount of parenchymal plaques by altering perivascular drainage of A\(\beta\) from the brain along cerebrovascular basement membranes (24). We have discovered several factors involved in perivascular drainage of A\(\beta\), including apolipoprotein E (ApoE), aging, and high-fat diet (25–27). Therefore, uncovering additional factors that contribute to A\(\beta\) clearance by any means is critical to further our understanding of how A\(\beta\) plaque and CAA levels are regulated.

Clusterin (CLU), also known as apolipoprotein J (apoJ), is a multifaceted protein that regulates a broad range of biological processes, including lipid metabolism (28–30), apoptosis (31), spermatogenesis (32), and aggregation and adhesion of cells (33). The single CLU gene, located on chromosome 8 in humans (34, 35), encodes a 70- to 80-kDa highly glycosylated protein that is cleaved to form \(\alpha\)- and \(\beta\)-subunits linked together by disulfide bonds during maturation (36, 37). With a central role in scavenging and survival (38, 39), the secreted form of CLU is a prominent chaperone in extracellular compartments (37). However, it has previously been reported that nuclear forms of CLU also exist from alternative splicing omitting exon 2 or translation from an alternative ATG start codon, although this is unique to the human transcript. Notably, nuclear CLU has been shown to trigger apoptosis in the cells under pathological conditions (40, 41).

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CLU is ubiquitously expressed in most mammalian tissues (29, 42–44), with the highest expression level in the CNS (45–47). For over two decades the e4 allele of APOE has been recognized as a major risk factor for both AD and CAA development (48, 49). However, the role of CLU, another abundantly expressed apolipoprotein in the brain (50), in Aβ pathology has received significantly less attention. Importantly, the levels of CLU have been found to be significantly elevated in AD patients compared with nondemented elderly individuals (51). Moreover, in vitro studies have shown that CLU directly interacts with Aβ (52) and facilitates the formation of toxic Aβ fibrils (53, 54). Such a role of CLU in amyloid pathology has been supported by multiple in vivo studies showing a profound effect of CLU on Aβ aggregation and toxicity (55, 56), as well as Aβ transport across the blood–brain barrier (BBB) (57–59).

In addition to functional studies supporting the role of CLU in AD, genome-wide association studies (60–63) have previously shown that genetic allelic variance in CLU SNPs are significantly associated with AD risk. More recently, rare CLU variants associated with AD have also been identified (64). Although a previous study using a transgenic mouse model of AD (PDAPP model) investigated the role of CLU in amyloid plaque formation, the effect of CLU on Aβ metabolism and deposition in cerebral vessels was not examined (56). Here, we used the well-characterized APP/presenilin 1 (PS1) mouse model of AD amyloidosis crossed to Clu knockout (Clu−/−) mice on a pure C57BL/6J background and conducted comprehensive histological and biochemical analyses.

Our findings have demonstrated that loss of CLU led to abundant CAA but simultaneously reduced brain parenchymal amyloid deposits. Despite the dramatic increase in CAA, the APP/PS1:Clu−/− mice presented with a significantly lower number of spontaneous hemorrhages and an overall decrease in inflammation and neuritic dystrophy compared with APP/PS1:Clu+/+ littermates. Importantly, we have provided in vivo evidence that loss of CLU is sufficient to alter the efficiency of the Aβ clearance from the brain. Finally, the presence of exogenous CLU decreased the amount of Aβ40 and Aβ42 associated with cerebrovasculature in ex vivo binding experiments, suggesting that in the absence of CLU the clearance of Aβ shifts to more perivascular drainage but results in the deposition of amyloid in the vessel walls as CAA, because of loss of CLU chaperone function. Taking these data together, this study suggests a novel role for CLU in mediating perivascular clearance of Aβ from the brain but also indicates that therapeutic targeting of CLU might unintentionally shift pathology to CAA.

**Results**

**CLU Colocalizes with Plaques and CAA and CLU Expression Determines Amyloid Distribution During Pathological Accumulation of Aβ.** To examine the impact of CLU on amyloid pathology, we first investigated the pattern of CLU colocalization with Aβ deposits in brain parenchyma and cerebrovasculature in APP/PS1 transgenic mice (65). In this mouse model there is rapid Aβ accumulation in the brain and development of CAA-associated hemorrhage (66). CLU immunostaining with the Congo red derivative X-34 counterstaining to label fibrillar amyloid revealed intense labeling of CLU with a “halo-like” appearance surrounding amyloid plaques in the brain parenchyma (Fig. 1A). CLU also extensively colocalized to Aβ deposits in cerebral blood vessels in APP/PS1 mice (Fig. 1A). In addition, CLU showed association with Aβ deposits

![Fig. 1. CLU expression influences Aβ pathology associated with AD in cortex and hippocampus of 12-mo-old APP/PS1 mice.](image-url)
in human cortex from an AD case with complete colocalization with CAA (Fig. 1B).

We then set out to determine whether changes in CLU levels influenced Aβ accumulation in the brain. We bred APP/PS1 mice onto a Clu+/+ or a Clu−/− background (littermates on C57Bl6/J background strain) and harvested PBS-perfused brains at 6 and 12 mo of age. Immunohistochemical analysis of Aβ and thioflavine-S staining revealed that CLU loss did not impact the onset of Aβ deposition in the brain but substantially influenced where Aβ accumulated. Specifically, 6- and 12-mo-old APP/PS1;Clu+/+ mice showed Aβ deposition mostly in the form of parenchymal plaques observed in the cortex (Fig. 1C and Fig. S1A) and hippocampus (Fig. 1D and E and Fig. S1 B and C), whereas in APP/PS1;Clu−/− mice, Aβ was predominantly deposited in the cerebrovasculature as CAA (Fig. 1F–H and Fig. S1 D–F). To more thoroughly analyze this dramatic shift in Aβ localization, we performed an unbiased stereological quantification of thioflavine-S deposition in brain parenchyma and cerebrovasculature in 6- and 12-mo-old mice (Fig. 2 and Fig. S2). We observed a highly significant reduction in the amount of thioflavine-S plaques in 6-mo-old APP/PS1;Clu−/− mice in the cortex (P < 0.0001) (Fig. S2A) and hippocampus (P < 0.05) (Fig. S2A) compared with control APP/PS1;Clu+/+ littermates. The absence of CLU also caused an increase in thioflavine-S Aβ accumulation in leptomeningeal vessels (P < 0.05 in the cortex and P < 0.05 in the hippocampus) (Fig. S2B) and penetrating arterioles (P < 0.05 in the cortex and hippocampus) (Fig. S2C) at 6 mo of age. Similarly, 12-mo-old APP/PS1;Clu−/− mice also showed reduced thioflavine-S deposition in parenchymal plaques (P < 0.0001 in the cortex and hippocampus) (Fig. 2A) and increased CAA in leptomeningeal vessels of the cortex (P = 0.062) (Fig. 2B) and hippocampus (P < 0.01) (Fig. 2B) and penetrating arterioles (P < 0.001 in the cortex and P < 0.05 in the hippocampus) (Fig. 2C). In addition, the ratio of CAA to amyloid plaques was significantly increased in these brain regions in 12-mo-old APP/PS1;Clu−/− mice compared with APP/PS1;Clu+/+ mice (Fig. 2D). Quantitatively, we observed a 40-fold and 6-fold increase in the ratio of CAA to parenchymal amyloid load in the cortex (P < 0.0001) and hippocampus (P < 0.0001), respectively, of 12-mo-old animals (Fig. 2D).

**Fig. 2.** Stereological quantification of amyloid deposition in brain parenchyma and cerebrovasculature in 12-mo-old APP/PS1 mice. (A) Twelve-month-old APP/PS1;Clu−/− mice had a significant decrease in the amount of amyloid plaques in cortex and hippocampus. (B) Significant increase in amyloid in leptomeningeal vessels and (C) penetrating arterioles was observed in the absence of CLU in cortex and hippocampus. (D) The ratio of CAA to thioflavine-S plaques was significantly increased in 12-mo-old APP/PS1;Clu−/− mice. (E) Twelve-month-old APP/PS1;Clu−/− mice also showed a decrease in the amount of total Aβ in the brain parenchyma in cortex and hippocampus. (F) The ratio of thioflavine-S amyloid plaques to total Aβ was not different between CLU genotypes in the cortex and hippocampus. Thioflavine-S (thio-S) was used to visualize fibrillar amyloid. n = 11–13 mice per group. For each animal three brain sections were analyzed. Data are presented as mean ± SEM and analyzed by Mann–Whitney test; *P < 0.05, **P < 0.01, ****P < 0.0001, N.S., not significant.
Similarly, at 6 mo of age, the ratio of CAA to amyloid plaques was a 50-fold increase in the cortex (P < 0.0001) and a 5-fold increase in the hippocampus (P < 0.01) (Fig. S2D). Numerous small vessels of the hippocampus were thioflavine-S positive. (Figs. S3 and S4). We observed significant differences in amyloid (Fig. 2F) and diffuse (thioflavine-S positive) plaque levels in 6- and 12-mo-old APP/PS1; Clu+/- mice compared with APP/PS1; Clu-/- mice. Additionally, the ratio of fibrillar plaques to total Aβ did not differ between Clu genotypes (Fig. 2F and Fig. S2F), with the exception of the cortical region of 6-mo-old animals, which showed a significant reduction in this ratio in APP/PS1; Clu+/- mice in relation to APP/PS1; Clu-/- mice (P < 0.0001) (Fig. S2F). Finally, we evaluated sex-dependent effects of Clu on amyloid pathology in 6- and 12-mo-old mice (Figs. S3 and S4). We observed significant differences in amyloid plaque formation (Figs. S3E and S4A and E) and CAA in penetrating vessels (Figs. S3G and S4 C and G) in 6- and 12-mo-old animals, with females having significantly more Aβ deposition in brain parenchyma and vasculature, suggesting a sex-associated increase in the severity of pathological presentation.

CLU Expression Alters Soluble and Insoluble Aβ Levels. Because Clu expression significantly impacts where Aβ deposits in the brain, we next examined whether the Clu genotype alters the levels of extractable forms of Aβ. ELISA was used to analyze insoluble (guanidine-HCl fraction, GDN) as well as TBS-soluble and detergent-soluble (TBS with Triton X-100, TBS-X) forms of Aβ40 and Aβ42 from the cortex and hippocampus of 6- and 12-mo-old APP/PS1; Clu+/- and APP/PS1; Clu-/- mice (Fig. 3 and Fig. S5). In both APP/PS1; Clu+/- and APP/PS1; Clu-/- mice, substantially higher concentrations of Aβ40 and Aβ42 were found in the insoluble fraction relative to soluble Aβ forms within each genotype (Fig. 3 and Fig. S5), reflecting that the majority of Aβ is deposited as insoluble parenchymal plaques and CAA, respectively. Relative to controls, APP/PS1; Clu-/- mice showed significantly lower levels of Aβ40 and Aβ42 in the GDN fraction from the cortex at 6 and 12 mo of age (P < 0.0001) (Fig. 3A and Fig. S5A) and Aβ42 from the hippocampus at 6 and 12 mo of age (P < 0.01 and P < 0.05) (Fig. 3D and Fig. S5D). Hippocampal levels of insoluble Aβ42 were not statistically different between CLU genotypes (Fig. 3D and Fig. S5D). Similarly, TBS and TBS-X soluble fractions showed dramatic reduction of Aβ40 and Aβ42 levels in the cortex of 6- [P < 0.01 and P < 0.0001 (Fig. S5B) and P < 0.0001 (Fig. S5C)] and 12-mo-old [P < 0.0001 (Fig. 3B) and P < 0.0001 (Fig. 3C)] APP/PS1; Clu-/- mice in relation to control APP/PS1; Clu+/- mice. Additionally, we found that hippocampal concentrations of soluble Aβ40 and Aβ42 of 12-mo-old mice APP/PS1; Clu+/- [P < 0.05, P < 0.0001 (Fig. 3E) and P < 0.01, P < 0.0001 (Fig. 3F)] and Aβ42 of 6-mo-old mice APP/PS1; Clu-/- [P < 0.01 (Fig. S5E) and P < 0.01 (Fig. S5F)] were significantly decreased relative to APP/PS1; Clu+/- controls. These data indicate that Clu expression alters the biochemical levels of Aβ deposition and are in agreement with the histological results.

Loss of Clu Significantly Reduces Parenchymal Plaque Load and Neuritic Dystrophy. Previous studies using AD mouse models have shown that severely dystrophic neurites surround fibrillar thioflavine-S positive plaques in the brain parenchyma in a Clu-dependent manner (56). To determine whether the Clu genotype affects neuritic dystrophy, we performed double-labeling of brain sections with lysosomal-associated membrane protein 1, (Lamp1), to mark dystrophic neurites, and thioflavine-S, to define fibrillar plaques (Fig. 4 A–C)
and Fig. S6 A–C). As expected, we found numerous dystrophic neurites around parenchymal plaques in 6- (Fig. S6A) and 12-mo-old APP/PS1;Clu−/− mice (Fig. 4A) but none observed in proximity to CAA alone (Fig. 4B and Fig. S6B). APP/PS1;Clu−/− mice had a significant reduction in the amount of fibrillar thioflavine-S plaques and a corresponding reduction in the overall amount of neuritic dystrophy compared with APP/PS1;Clu+/+ mice (Fig. 4B and D). However, although CLU has previously been reported to dissociate neuritic dystrophy from fibrillar amyloid plaques (56), we found no evidence of reduced neuritic dystrophy surrounding the few fibrillar thioflavin-S plaques that were detected in APP/PS1;Clu−/− mice (Fig. 4C and Fig. S6C). The discrepancy between our results and previous reports may be because of differences in the APP transgenic model or the mixed genetic background of Demattos et al. (56), which also raises the possibility that other genetic modifiers are present that mediate the amyloid associated neuritic dystrophy.

**Despite Increases in CAA, Absence of CLU Reduces Hemorrhage and Neuroinflammation Associated with Aβ Pathology.** CAA is known to cause cerebral hemorrhage in AD patients (24). To examine if the elevated CAA observed in APP/PS1;Clu−/− mice was also associated with increased occurrence of cerebral hemorrhage, Prussian blue staining was conducted on 12-mo-old APP/PS1;Clu+/+ and APP/PS1;Clu−/− mice (n ≥ 18 sections per mouse spaced 300-μ apart). APP/PS1 mice predominantly develop microhemorrhages in the cortex and hippocampus because these two regions are the most severely affected by CAA. Despite the fact that they had substantially increased CAA, we found that APP/PS1;Clu−/− mice had significantly fewer spontaneous microhemorrhages compared with control APP/PS1;Clu+/+ mice (P < 0.05) (Fig. 5A). When microhemorrhages were normalized to CAA load, we observed an even greater disparity between APP/PS1;Clu+/+ and APP/PS1;Clu−/− mice (P < 0.001) (Fig. 5B).

![Image](image.png)

**Fig. 4.** Absence of CLU reduces the total amount of neuritic dystrophy in APP/PS1 mice. (A) In APP/PS1;Clu+/+ mice all parenchymal plaques (green) were surrounded by dystrophic neurites (red) identified with Lamp1 antibody. (B) APP/PS1;Clu−/− mice had an overall reduction in the amount of parenchymal plaques and a concomitant reduction in total neuritic dystrophy. Arrowheads indicate CAA. (Scale bars in A and B, 50 μm) (C) However, prominent neuritic dystrophy was seen around parenchymal plaques that do form in APP/PS1;Clu−/− mice. (Scale bars, 20 μm.) (D) Quantification of Lamp1 intensity in brain sections of 12-mo-old APP/PS1 mice. n = 8–12 mice per group. Data are presented as mean ± SEM and analyzed by Mann–Whitney test; ***(P < 0.001.

Given that both parenchymal plaques and CAA are independently associated with neuroinflammation (67), we next investigated whether the CLU genotype had a differential effect on gliosis. Abundant astrogliais was present around amyloid plaques in brain parenchyma of APP/PS1;Clu−/− mice (Fig. 5C). Although the absence of CLU resulted in a dramatic increase in CAA, the level of astrogliais was significantly reduced when assessed at 12 mo of age in APP/PS1;Clu−/− mice (Fig. 5D and E). Similarly, APP/PS1;Clu−/− mice had significantly decreased microgliosis compared with APP/PS1;Clu+/+ mice (Fig. 5 D and F). Reactive astrocytes and microglia were not observed in proximity to CAA regardless of CLU genotype (Fig. 5D). To test whether CLU genotype also affected neuroinflammation at the molecular level, we profiled inflammatory cytokine transcripts, Tnfa and Il6, by real-time quantitative PCR. The levels of Tnfa and Il6 were significantly increased in APP/PS1;Clu+/+ compared with nontransgenic Clu+/+ littermates, but these levels were significantly reduced to near baseline (nontransgenic levels) in APP/PS1;Clu−/− mice (Fig. 5G and H). Taken together, these experiments demonstrate that the majority of inflammation in the presence of amyloid is because of parenchymal plaques rather than CAA, at least in the absence of CLU.

**CLU Does Not Impact APP Processing or Cause Widespread Transcriptional Changes in Known Aβ Metabolism Pathways.** The intriguing association between loss of CLU and dramatic increase in CAA led us to test whether CLU alters APP metabolism. We performed Western blot analysis to assess the level of full-length APP and soluble APPα (sAPPα) in brain homogenates of APP/PS1;Clu+/+ and APP/PS1;Clu−/− mice (Fig. S7A). The CLU genotype did not alter APP and sAPPα expression levels, indicating that CLU does not grossly affect APP processing (Fig. S7 B and C). A myriad of other factors besides APP processing could explain the shift in Aβ pathology from parenchymal plaques to...
CLAU. We therefore sought to determine whether specific CLU-dependent changes occurred in the brain transcriptome that might explain this shift in pathology. To identify differentially expressed transcripts between CLU genotypes, we performed an RNAseq transcriptomic study of whole-brain tissue from 6-mo-old Clu−/− and Clu+/+ mice (n = 4 per genotype). However, this analysis yielded only four protein-coding transcripts that were differentially expressed after false-discovery rate correction between Clu−/− and Clu+/+ mice, including Clu itself, Slc2a5, Hprt, and Frem1 (Table S1). No significant changes were found in other AD genes, such as Apc4, Bin1, Abca7, Picalm, Cld33, Cd2ap, or any of the several putative Aβ degrading enzymes (Table S2). It has previously been shown that overexpression of Tgfb1 in APP transgenic mice results in a shift in Aβ pathology from parenchymal to vessels (68), but our transcriptome study did not show any significant changes in Tgfb1 or the TGF-β pathway in general. These findings suggest that CLU-deficiency itself does not significantly impact the whole-brain transcriptome and that the effects seen on Aβ deposition are likely direct in nature.

**CLU Alters Aβ Clearance Pathway and Prevents Ex Vivo Binding of Aβ to Isolated Cerebrovasculature.** To gain insight into the possible mechanism underlying the dramatic shift in the Aβ deposition from parenchyma to cerebrovasculature in APP/PS1;Clu−/− mice, we used in vivo microdialysis (Fig. 6 A–C). Because soluble Aβ in the interstitial fluid (ISF) has been shown to correlate with Aβ deposition in the brain parenchyma (69), we measured the hippocampal steady-state level in 10-wk-old APP/PS1;Clu−/− and APP/PS1;Clu+/+ mice. To determine whether CLU genotype had a differential effect on Aβ clearance, we infused a potent γ-secretase inhibitor that rapidly blocked Aβ production, therefore allowing us to examine the half-life (t1/2) of Aβ40. The concentration of hippocampal Aβ40, measured in ISF, gradually decreased over time, with APP/PS1;Clu+/+ showing faster decline compared with APP/PS1;Clu−/− mice (Fig. 6B). Moreover the t1/2 of ISF Aβ40 was significantly longer in mice lacking CLU compared with control littermates (P < 0.05) (Fig. 6C). These results suggest that the loss of CLU may alter the clearance of soluble Aβ from the ISF.

Numerous studies have previously shown that Aβ40 is predominantly present in vascular amyloid because of its more soluble nature (70, 71), whereas Aβ42, a more fibrilligenous form of Aβ, is mainly found in parenchymal amyloid (72, 73). Therefore, the ratio of Aβ40:42 seems to determine where Aβ deposits in the brain with higher Aβ40:42 ratio predisposing the formation of CAA (74). In agreement with this hypothesis, we found a slight increase in the Aβ40:42 ratio (P = 0.057) (Fig. 6D) in APP/PS1; Clu−/− mice compared with APP/PS1;Clu+/+ mice.

Given that CAA and AD appear to result from a disruption of the perivascular drainage pathway (24, 75), we sought to investigate the potential role of CLU in the Aβ removal along the
0.01 (Fig. 6c) cerebral blood vessels compared with samples lacking exogenous
To this end, we freshly isolated cerebral vessels using density-binding of Aβ in the hippocampus of 10-wk-old APP/PS1;Clu<sup>−/−</sup> and APP/PS1;Clu<sup>+/+</sup>. (A) The concentration of Aβ<sub>40</sub> in ISF was measured as the basal level of Aβ. (B) The logarithm (log) of percentage baseline ISF Aβ<sub>40</sub> versus time was plotted after treatment of mice with a potent γ-secretase inhibitor. (C) The slope from the linear regressions from log (percent ISF Aβ<sub>40</sub>) was used to assess the half-life of Aβ<sub>40</sub> elimination from the ISF. **<i>P</i> < 0.01. (D) Data were presented as ± SEM and analyzed by Student’s t test; *<i>P</i> < 0.05. (E) The ratio of Aβ<sub>40</sub> to Aβ<sub>42</sub> was examined from 10-wk-old APP/PS1;Clu<sup>−/−</sup> and APP/PS1;Clu<sup>+/+</sup>. (F) The number of arteries with colocalization of fluorescently labeled human Aβ<sub>40</sub>. Fewer arteries with Aβ colocalization were observed in Clu<sup>−/−</sup> mice compared with Clu<sup>+/+</sup> animals, (F and G) Exogenous human Aβ<sub>40</sub> and (G) Aβ<sub>42</sub> were applied to isolated cerebral vessels with and without exogenously added CLU and binding of Aβ was measured by ELISA. The addition of 1 μM exogenous CLU led to a significant reduction in the levels of (F) Aβ<sub>40</sub> and (G) Aβ<sub>42</sub> associated with cerebrovasculature. (H and I) Isolated vessels were treated with increasing concentrations of exogenous exogenous CLU, (H) Aβ<sub>40</sub> Clu<sup>+/−</sup> and (I) Aβ<sub>42</sub> in the absence and presence of increasing concentrations of CLU. CLU addition resulted in a decrease amount of (H) Aβ<sub>40</sub> and (I) Aβ<sub>42</sub> bound to cerebral vessels even at high Aβ concentrations. Data presented as ELISA replicates. n = 3-4 mice per group. Data are presented as ± SEM and analyzed by Student’s t test; **<i>P</i> < 0.01.

These findings led us to evaluate whether there was a direct effect of CLU on CAA by measuring the ability of CLU to alter binding of Aβ to the cerebrovasculature in an ex vivo microdialysis assay. To this end, we freshly isolated cerebral vessels using density-mediated separation to purify vessels from parenchymal components, as previously described (76). Vessels were then treated with exogenous human Aβ<sub>40</sub> or Aβ<sub>42</sub> in the presence or absence of exogenous CLU, then washed, lysed in GDN buffer, and Aβ levels were assessed by ELISA. We found that addition of exogenous CLU resulted in a significant reduction of the amount of Aβ<sub>40</sub> (**<i>P</i> < 0.01) (Fig. 6F) and Aβ<sub>42</sub> (P < 0.01) (Fig. 6G) bound to isolated cerebral blood vessels compared with samples lacking exogenous CLU. A similar effect was observed when isolated cerebral vessels were treated with increasing concentrations of exogenous Aβ<sub>40</sub> or Aβ<sub>42</sub> in the presence of equally increasing concentrations of exogenous CLU (Fig. 6H and I). The addition of exogenous CLU led to a dramatic decrease of the level of Aβ associated with the cerebrovasculature compared with vessels without CLU added, even when assessed at high Aβ concentrations. Taken together, these results suggest that in the absence of CLU, Aβ clearance shifts to perivascular drainage, resulting in decreased parenchymal amyloid but also in the aggregation and deposition in the cerebral blood vessels because of loss of CLU chaperone activity.

**Discussion**

In the present study, we investigated whether alterations in CLU expression affect amyloid-driven pathology. Using the APP/PS1 mouse model of AD amyloidosis, we showed that in sharp contrast to the abundant brain parenchymal amyloid plaque accumulation and minimal CAA observed in APP/PS1; Clu<sup>−/−</sup> mice, APP/PS1;Clu<sup>+/−</sup> mice had few parenchymal plaques but robust CAA, even when assessed at a young age. In addition, CLU loss resulted in substantial alterations of dynamic pools of soluble and insoluble Aβ. We further demonstrated that lack of CLU significantly reduced the number of
CAA-associated microhemorrhages, despite the fact that the APP/PS1; Clu−/− mice had a tremendous elevation in the amount of CAA. Our in vivo data also showed that APP/PS1; Clu+/− mice exhibited significantly less neuritic dystrophy and reduced cellular and molecular inflammation compared with APP/PS1; Clu−/− mice. Importantly, by using in vivo microdialysis, we provided evidence that CAA is involved in the elimination of Aβ from the brain. Consistent with this notion, intracerebral injections of Aβ40 of young Clu+/+ and Clu−/− mice resulted in a decreased number of arteries with fluorescence labeled Aβ40, implying the disruption of perivascular drainage pathway in the absence of CAA. Finally, we identified that the presence of exogenously added CAA reduced binding of Aβ40 and Aβ42 to isolated cerebral vessels, suggesting that CAA impacts Aβ pathology in vessels by preventing it from binding and aggregating during ISF drainage.

Growing evidence suggests that CAA is an important player in Aβ deposition, fibrillogenesis, and clearance (53, 55–57). The in vivo consequences of CAA loss were previously assessed in the PDAPP mouse model of AD (55, 56). These seminal reports showed that absence of CAA was associated with a substantial reduction of fibrillar amyloid plaques but no change in total Aβ deposition in brain parenchyma. Our data are in agreement with the effect of CAA on fibrillar plaques but, in contrast, we found that the absence of CAA also reduced total Aβ load.

One of the most striking phenotypes of CAA loss in our AD amyloidosis model was the shift in the localization of Aβ deposition from parenchymal plaques to CAA. Although DeMattos et al. (56) did not directly analyze CAA levels in their study, such an obvious pathology would have been readily noticed. Therefore, the differences in these studies likely reflect the different APP transgenic models used (PDAPP vs. APP/PS1) or the mixed genetic background of the PDAPP mouse model.

In light of increasing evidence that disruption of Aβ clearance mechanisms from the brain initiates the pathogenic cascade of AD (23), identifying factors that contribute to Aβ elimination is critical. Importantly, we showed that the loss of CAA is sufficient to reduce the efficiency of Aβ clearance in the hippocampus in our mouse model of AD amyloidosis. In agreement with this observation, we found an increased Aβ40:42 ratio in APP/PS1; Clu−/− mice, possibly contributing to the shift of Aβ deposition between brain compartments. Given that Aβ42 appears to be more amyloidogenic in cerebral vessels (70, 71), whereas Aβ40 is thought to be a predominant form present in the brain parenchyma (72, 73), Aβ40:42 ratio might be an important factor in determining where Aβ deposits.

In fact, several lines of evidence have previously suggested that a high Aβ40:42 ratio favors the development of CAA (74, 77). The APP Dutch animal model, which recapitulates the characteristics of hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) and shows Aβ accumulation predominantly in the cerebral vessels, appears to have a highly elevated Aβ40:42 ratio compared with animals overexpressing human wild-type APP (77). In addition, it has been reported that Tg2576 mice expressing human ApoE4 develop CAA, which is also likely attributable to the higher ratio of Aβ40:42 in these animals in relation to animals expressing endogenous murine ApoE (74). In contrast, a lower Aβ40:42 ratio seems to promote amyloid deposition in brain parenchyma versus cerebrovasculature. It has been shown that APP mice harboring the “Indiana” mutation, which leads to the highly elevated levels of Aβ40, have a reduction in Aβ40:42 ratio, and therefore mainly parenchymal deposition of Aβ (78). This notion is further supported by observation that PDAPP mice lacking ApoE have an increased production of Aβ40, which results in deposition of parenchymal amyloid with very minimal CAA (76).

Among numerous Aβ clearance pathways in the brain that have previously been described (57, 68, 79–84), perivascular drainage along basement membranes of cerebral arteries is one of the major routes for Aβ removal and its impairment leads to CAA formation (25, 26). We found a reduced number of arteries with colocalization of injected fluorescent Aβ in the basement membranes in Clu−/− mice compared with control littermates, suggesting the disruption of perivascular drainage of Aβ in the absence of CAA. Consistent with this notion, we found direct ex vivo evidence that CAA alters Aβ binding to isolated cerebral vessels, which might exacerbate development of CAA. Therefore, we propose that it is CAA facilitates Aβ clearance along ISF drainage pathways by preventing binding to cerebral vessels, possibly through the interactions with cerebrovascular basement membrane components. Thus, as a consequence of CAA loss, Aβ fibrils accumulate in the cerebral vessels and lead to CAA.

Interestingly, using unbiased proteomic analysis, we have recently demonstrated that the level of CAA protein is significantly elevated in human leptomeningeal arteries with CAA (85), suggesting the entrapment of the Aβ-CAA complex in the perivascular drainage pathways, or a compensatory up-regulation of CAA to clear Aβ.

Despite the evidence that loss of CAA leads to the accumulation of Aβ in the walls of cerebral vessels, possibly mediating the formation of CAA, we cannot rule out the possibility that other mechanisms also contribute to Aβ deposition in different brain compartments. Previous reports have demonstrated that the transport of soluble Aβ across the BBB can be facilitated via low-density lipoprotein receptor-related protein 1 (LRP1) (84). In addition, the low-density LRP2 has been previously shown to mediate the elimination of Aβ from the brain. LRP2 is a receptor for CAA localized at the BBB and it has been suggested to be essential for the transport of the Aβ-CAA complex into circulation (57). It is possible that the absence of CAA also disrupts the Aβ transport across the BBB via LRP2, leading to the accumulation of Aβ within the walls of the cerebrovasculature. Although the BBB plays a significant role in the Aβ clearance, whether and to what extend BBB transporters contribute to the development of CAA in APP/PS1; Clu−/− mice is yet to be determined, although we found no evidence of altered transcript levels of Lpl1, Lpl2, or other members of the LDLR family in our RNAseq data.

Mounting evidence has demonstrated the strong association between CAA and cerebral hemorrhage in elderly individuals. Recent cerebral hemorrhage is also present in patients with hereditary cerebral hemorrhage with amyloidosis Icelandic type (HCHWA-I), however it is also frequently observed in individuals with sporadic CAA (86, 87). Several lines of evidence suggest that cerebral hemorrhage is caused by gradual smooth muscle cell degeneration in the walls of cerebral vessels, leading to their weakening and rupture (88). Spontaneous acute hemorrhage has also been linked to widespread Aβ deposition in leptomeningeal and cortical vessels in several transgenic mice. Winkler et al. showed that accumulation of Aβ is sufficient to give rise to recurrent hemorrhagic stroke in APP23 mice (89). Similar findings have been reported for other transgenic mouse models overexpressing human APP harboring various mutations, including Tg2576, PDAPP (76), TgSwDI (90), and APPDutch (77), which develop spontaneous hemorrhage in association with Aβ-laden vessels. Interestingly, the loss of ApoE in Tg2576 and PDAPP mice completely prevented CAA and hemorrhage, indicating that ApoE facilitates CAA and CAA-associated hemorrhage (76). Although CAA is a major risk factor for developing hemorrhage, we observed a significant decrease in the number of microhemorrhages in APP/PS1; Clu−/− mice compared with APP/PS1; Clu+/+ animals. A possible explanation for this difference with previous studies could be that CAA expression alters the structure or amount of amyloid deposited in the walls of cerebrovasculature, causing their damage.

It is recognized that neuroinflammation is another component commonly observed in individuals with CAA (91). Similar to human studies, Herzig et al. have observed that an inflammatory response is associated with vascular amyloid and exists independently from amyloid plaques in APPDutch mice (77). Miao
et al. have shown that reactive astrocytes and activated microglia were present in vicinity of Aβ-laden vessels in Tg-SwDI transgenic mice (92). In addition, elevated levels of inflammatory cytokines, including IL-6 and IL-1β, were noted in these animals (92). Although these studies support an association of vascular amyloid with neuroinflammation, the majority of CAA in these models is weighted toward capillaries. Our data indicate that the cellular and molecular inflammation are more associated with parenchymal amyloid load rather than CAA. These observations raise the possibility that CAA, as seen in sporadic CAA, is not sufficient to cause neuroinflammation in APP/PS1 mice or that the combination of CAA and CLU expression is critical for induction of inflammatory response. Additional studies are needed to further address this issue.

Given the role of CLU in Aβ accumulation, transport, and toxicity, and its strong genetic association with AD, we aimed to elucidate how CLU affects Aβ pathology and discovered a role in the pathophysiology of both parenchymal plaque formation as well as CAA. Future studies are crucial to gain a detailed view of additional mechanisms underlying the role of CLU in CAA and to better understand specific events leading to pathogenesis of AD and CAA. This could allow optimization of therapeutic strategies to limit Aβ deposition in brain parenchyma and cerebrovasculature. Therapeutics that intentionally or unintentionally decrease the levels of CLU may result in an unwanted shift of Aβ pathology to CAA, although our data indicate that the brain may be more tolerant of amyloid in the cerebrovasculature than in the parenchyma.

Materials and Methods

Animals. APP/PS1 mice bearing a double-mutation APPsw/PS1+42/69 were used (65). All studies were done in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals (93) under an approved protocol from the Mayo Clinic Institutional Animal Care and Use Committee. De-identified postmortem, pathologically confirmed AD brain tissue were obtained through the Mayo Clinic Brain Bank for neurodegenerative diseases, whose operating procedures are approved by the Mayo Institutional Review Board.

Histopathological Analyses. PBS-perfused brains from APPPS1;Cla+/+ and APP/PS1;Cla−/− mice were used and analyzed using a Zeiss Axioslager.Z1/Apo-Tome microscope. Aβ pathology was quantified, as previously described (76).

Biochemical Analyses. Cortex and hippocampus were dissected from APPPS1;Cla+/+ and APP/PS1;Cla−/− PBS-perfused brains. Separate extraction for each condition was used. Aβ42 and Aβ40 levels were assessed by ELISAs. To examine APP processing, the cortices of APPPS1;Cla+/+ and APPPS1;Cla−/− mice were used. Total RNA was isolated using a Total Aurum RNA isolation kit. Random-prime reverse transcription was performed. All samples were run on an ABI 7900 HT Fast Real-Time PCR instrument.

In Vivo Clearance. In vivo microdialysis in APPPS1;Cla+/+ and APPPS1;Cla−/− mice was performed, as described previously (69, 94). Perivascular drainage was quantified in Clu+/+ and Clu−/− mice, as described previously (25).

Aβ Binding to Cerebrovasculature. Cerebral vessels were isolated from C57BL/ 6J mice, as described previously (76). Vessels were treated with Aβ42 or Aβ40 with or without CLU. The Aβ binding to vasculature was assessed by ELISA.

Statistical Analyses. For all statistical analyses GraphPad Prism 5.04 software was used. For additional descriptions of methods, please see SI Materials and Methods.

ACKNOWLEDGMENTS. J.D.F. was supported by the Mayo Foundation, the GHR Foundation, the Mayo Clinic Center for Individualized Medicine, a Mayo Clinic Gerster Family Career Development Award, the Ed and Ethel Moore Alzheimer’s Disease Research Program of Florida Department of Health (6AZ06), the Gilmer Family Foundation, Alzheimer’s Association NRP- 12-25928, and NIH Grants NS04137, AG047327, and AG049992. S.S.K. was supported by The Robert and Clarice Smith and Abigail Van Buren Alzheimer’s Disease Research Program Fellowship, the Mayo Clinic Program on Synaptic Biology and Memory, and NIH Grant MH03632. G.B. was supported by NIH Grants AG027924, AG035355, and NS027496.

Supporting Information

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SI Materials and Methods

**Animals.** APP/PS1 mice bearing a double mutation in APP and PS1 (APPSwe/PS1ΔE9) were used (65). These mice have been extensively backcrossed with C57BL/6J mice and were used to generate the mice used in this study using a littermate breeding strategy of CluΔE9 mice bred to APP/PS1;CluΔE9 mice. All studies were done in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals (93) under an approved protocol from the Mayo Clinic Institutional Animal Care and Use Committee. Analyses were performed included mice of both sexes in accordance with NIH directives.

**Histopathological Analyses.** To examine CLU association with CAA in humans, a brain specimen was obtained from Mayo Clinic Brain Bank from an individual diagnosed with AD and with the presence of CAA. Paraffin-embedded sections were first cut at 10 μm, then deparaffinized, rehydrated, and subjected to antigen-retrieval in dH2O for 15 min under high temperature. Brain sections were incubated overnight with goat anti-CLU antibody (1:25; Santa Cruz) diluted in 0.5% dry-milk in PBS, washed three times in Triton X-100 in PBS (PBS-X) and PBS, followed by overnight incubation with Alexa Fluor-488-conjugated secondary antibody (1:250; Jackson ImmunoResearch). Sections were double labeled with the X-34 stain to detect fibrillar amyloid and mounted using VECTASHIELD (Vector Laboratories). The images were captured using ZEISS AXIOIMAGER.Z1/APOTOmE microscope.

For histopathological analyses, 6- and 12-month-old APP/PS1; CluΔE9 and APP/PS1;CluΔE9 mice were used. Animals were anesthetized with pentobarbital (100 mg/kg, i.p.) and transcardially perfused with PBS followed by overnight immersion of one hemibrain in 10% phosphate buffered formalin at 4 °C. The hemibrain was then transferred to 30% sucrose overnight and cut into 50-μm coronal sections using a freezing-sliding microtome. Briefly, brain sections were permeabilized for 30 min in 0.3% PBS-X and blocked for 1 h in 1% dry-milk in PBS-X. Sections were incubated overnight with primary antibodies diluted in 0.5% dry-milk in PBS. Goat anti-CLU antibody (1:25; Santa Cruz) was used to detect CLU.

To examine total brain amyloid deposition, sections were incubated with mouse anti-Aβ antibody (1:1,000; MOAB2, Abcam). Dystrophic neurites were detected by using an antibody to LAMP1 (1:500; Developmental Studies Hybridoma Bank) (95). Activated microglia and reactive astrocytes were examined by using, rabbit antionized calcium binding adaptor molecule 1, Iba1 (1:250; Wako Chemicals) and mouse antiglial fibrillary acidic protein (1:250; Cell Signaling Technology), respectively. After incubation with primary antibodies, brain sections were washed with PBS-X and PBS and incubated overnight with secondary antibodies. The following secondary antibodies were used: donkey anti-goat Alexa Fluor-488-conjugated secondary antibody (1:250; Jackson ImmunoResearch), Cy3-conjugated AffiniPure donkey anti-rabbit (1:250; Jackson ImmunoResearch), and goat anti-mouse Alexa Fluor-488-conjugated antibody (1:250; Jackson ImmunoResearch). To visualize fibrillar amyloid, brain sections were stained with thioflavine-S (Sigma) or X-34, generous gift from John R. Cirrito, Washington University in St. Louis, St. Louis, MO. Sections were mounted with VECTASHIELD (Vector Laboratories.). Images were acquired using a ZEISS AXIOIMAGER.Z1/APOTOmE microscope.

**Quantitative Analysis of Amyloid Plaque and CAA Load.** PBS-perfused brains from 6- and 12-mo-old APP/PS1;CluΔE9 and APP/PS1;CluΔE9 mice (with males and females balanced in each group) were used. For each animal, three sections separated by 300 μm were analyzed using unbiased stereological quantification as previously described (76). StereoInvestigator software (MBF Bioscience) was used to assess the percentage of area covered by total Aβ, fibrillar amyloid, and CAA presence in leptomeningeal and penetrating vessels in the cortex and hippocampus.

**Preparation of Brain Lysates.** For biochemical analysis, the cortex and hippocampus were dissected from APP/PS1;CluΔE9 and APP/PS1;CluΔE9 PBS-perfused brains at the ages of 6 and 12 mo (with males and females balanced in each group). Samples were snap-frozen on dry ice and stored at −80 °C until processing. Separate extraction for each condition was used to obtain soluble and insoluble fractions. Briefly, touch sonication was performed in TBS and lysates were centrifuged at 15,000 × g for 15 min at 4 °C. After centrifugation, supernatant was collected and the pellet was incubated in TBS with 1% of TBS-X for 1 h on ice followed by centrifugation at 15,000 × g for 15 min at 4 °C. The same step was repeated for 5 M guanidine-HCl extraction.

**Quantitative Analysis of Aβ by ELISA.** Aβ40 and Aβ42 levels were assessed by sensitive sandwich Aβ40 or Aβ42-specific ELISAs. Plates were coated using human anti-Aβ40 (13.1.1) and Aβ42 (2.1.3) capture antibodies. Aβ standards were prepared by using human synthetic Aβ40 and Aβ42. To detect Aβ species, HRP-conjugated Ab5 secondary antibody was used followed by the incubation with Super Slow ELISA TMB reagent (Sigma) to develop. Protein concentrations were assessed by Bicinchoninic Acid (BCA) Protein Assay kit (Thermo Scientific), according to the manufacturer’s instructions with a standard curve using BSA.

**Quantitative Analysis of Hemorrhage.** Hemorrhage occurrence was assessed in APP/PS1;CluΔE9 and APP/PS1;CluΔE9 mice at the age of 12 mo (with males and females balanced in each group) using Prussian blue staining. Briefly, brain sections of 50-μm thickness were incubated with 0.25% Triton X-100 in PBS to allow for permeabilization followed by 20-min incubation with 5% potassium ferrocyanide and 5% HCl. For each animal, a set of 18–21 sections spaced 300-μm apart was examined using light microscopy.

**Immunoblotting.** To examine APP processing, detergent-soluble fractions (TBS-X) from the cortical region of 6-month-old APP/PS1;CluΔE9 and APP/PS1;CluΔE9 mice were used. BCA assay was performed to determine protein concentrations. Ten micrograms of protein was loaded onto 4–12% SDS polyacrylamide gel followed by the transfer of the proteins to nitrocellulose membrane and overnight incubation with anti-APP (1:1,000; 6E10) primary antibody. The HRP-conjugated secondary antibody (1:5,000; Jackson ImmunoResearch) was applied for one hour and ECL reagent (Pierce ECL Western Blotting Substrate) was used to visualize specific bands.

**qPCR.** Total RNA was isolated using a Total Aurum RNA isolation kit according to the manufacturer’s instructions with column DNase I treatment (Bio-Rad). Random-primed reverse transcription was performed according to manufacturer protocols (Invitrogen-Life Technologies). cDNA was added to a reaction mix (10-μL final volume) containing 300-nM gene-specific primers and Universal SYBR green supermix (Bio-Rad). All samples were run in triplicate and were analyzed on an ABI 7900 HT Fast Real Time PCR instrument (Applied Biosystems–Life Technologies). Relative gene expression was normalized to GAPDH controls and assessed using the 2−ΔΔCT method. Primer
sequences are as follows (5’ to 3’): Gapdh F: CTGCACCACAATGCCTTGGAGTGA, Gapdh R: ACGAGTCGGGTTTCGTGGA, Ctna F: CCACCAGCTCTTGGGTGGCA, Ctna R: ATGAGGAGGAGCATTGG; Il6 F: TTCTATACACTTGCATACAAGTGGA, Il6 R: GAATTGCCATGGCACAACCTTCT.

**In Vivo Microdialysis.** To investigate whether CLU alters Aβ clearance, in vivo microdialysis in awake, freely moving 10-wk-old APP/PS1:Clu+/+ and APP/PS1:Clu−/− aniamas was performed, as previously described (69, 74). Briefly, under anesthesia a microdialysis probe (38-kDa molecular mass cut-off membrane; Bioanalytical Systems) was inserted into the hippocampus (3.1 mm behind Bregma, 2.5 mm lateral to midline, and 1.2 mm below dura at a 12° angle) through guide cannula (Bioanalytical Systems). Artificial cerebrospinal fluid (1.3 mM CaCl2, 1.2 mM MgSO4, 3 mM KCl, 0.4 mM KH2PO4, 25 mM NaHCO3, and 122 mM NaCl, pH 7.35) containing 4% BSA (Sigma) was used as a perfusion buffer. Flow rate was a constant 1.0 μL/min. Samples were collected every 60–90 min overnight, which gets through the 4- to 6-h recovery period. The mean concentration of Aβ over the 6-h preceding treatment was defined as basal concentration of ISF Aβ. Aβ40 was assessed by ELISA. To determine Aβ half-life, a γ-secretase inhibitor (LY411575; 5 mg/kg) was administered intraperitoneally. Microdialysis samples were collected every 60 min for 5 h. The Aβ half-life was calculated based on the slope of the semilog plot of percent change in Aβ versus time.

**Perivascular Drainage Pathway.** To determine the effect of Clu genotype on perivascular drainage of solutes, 3-mo-old Clu+/+ and Clu−/− mice (n = 3 per group) were stereotactically injected with 0.5 μL HiLyte Fluor-TR labeled human Aβ40 (the final concentration of injected Aβ40 was 100 μM; Cambridge Biosciences) into the corpus callosum (coordinates from Bregma: anterior-posterior = −2.2 mm; medio-lateral = −1.6 mm; dorso-ventral = −1.18 mm). As previously described (25), 10-min postinjection, mice were transcardially perfused with PBS followed by 4% paraformaldehyde (PFA). After overnight immersion in 4% PFA, the brain was transferred to 30% sucrose and stored at 4 °C until time of cutting. The brains were cut into 1.18 mm. As previously described (25), 10-min postinjection, mice were transcardially perfused with PBS followed by 4% paraformaldehyde (PFA). After overnight immersion in 4% PFA, the brain was transferred to 30% sucrose and stored at 4 °C until time of cutting. The brains were cut into coronal sections of 10-μm thickness using cryostat and mounted on slides. To quantify perivascular drainage, anticoagulant IV (1:500; Abcam) and anti-α-smooth muscle actin (1:500; Sigma-Aldrich) double-labeling was used for counting the number of arterries containing Aβ. Photomicrographs of double-labeling immunohistochemistry were captured from tissue sections at the injection site and 100-μm posterior using Leica SP8 confocal laser scanning microscope. The total number of arteries containing fluorescent Aβ40 was determined in each brain hemisphere. For each group at each site were calculated. Vessels were identified on the basis of anticonnect IV staining and morphology. Arteries were identified as having a diameter over 15 μm and anti-α-smooth muscle actin staining.

**Aβ Binding to Cerebrovasculature.** As previously described (76), 8-wk-old C57BL/6J mice were anesthetized with pentobarbital (100 mg/kg, i.p.) and transcardially perfused with PBS. Immediately after perfusion, the forebrain was dissected and placed in loose-fitting homogenizer with fivefold excess of ice-cold HBSS buffer supplemented with 15 mM Hepes, 1 mM pyruvate, 0.1% BSA, and 1% dextran (MW = 64,000, Sigma). The same volume of dH2O with 31% dextran was added and the tissue homogenate was aliquoted to microcentrifuge tubes and centrifuged at 8,000 × g for 30 min at 4 °C. The vessel material formed a pellet at the bottom while parenchymal material collected on top of the buffer. After removal of parenchymal material, the vessel pellet was washed four times in HBSS buffer supplemented with 0.5% BSA. After the last wash, the pellet was resuspended in the same buffer, passed through a 40-μm nylon mesh, and washed with a large amount of buffer. Vessels were then recovered by inverting the mesh and collected with a stream of buffer into a 50-mL conical tube and centrifuged at 1,000 × g for 5 min at 4 °C. Vessel pellets were transferred to a microcentrifuge tube and resuspended in HBSS buffer supplemented with 0.5% BSA and subjected to treatments with the following conditions: (i) 1 μM Aβ40 alone; (ii) 1 μM Aβ40 + 1 μM exogenous recombinant CLU (R&D Systems); (iii) 1 μM Aβ42 alone; and (iv) 1 μM Aβ42 + 1 μM exogenous recombinant CLU. The binding of Aβ to vasculature with or without CLU was performed for 30 min at room temperature with gentle rotation. Vessels were then washed three times in HBSS buffer supplemented with 0.5% BSA and lysed in 5 M guanidine-HCl for 1 h at room temperature. We further examined the Aβ40 binding to cerebrovasculature by using increasing concentrations of Aβ40 and Aβ42 ranging from 0.1 to 5 μM with increasing amount of recombinant CLU or without CLU followed by lysis in 5 M guanidine-HCl. The quantification of the Aβ binding to vasculature was assessed by ELISA. Protein concentrations were assessed by BCA.

**Statistical Analyses.** Statistical significance of experiments involving two groups was assessed by Mann–Whitney test when data were not normally distributed. For normally distributed data, Student’s t test was used. The statistical analyses of more than two groups were performed by using two-way ANOVA with the Tukey’s post hoc test. Data are presented as mean ± SEM. For all statistical analyses GraphPad Prism 5.04 software was used (GraphPad).

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Fig. S1. CLU expression influences Aβ pathology associated with AD in the cortex and hippocampus of 6-mo-old APP/PS1 mice. (A–C) APP/PS1;Clu+/+ mice developed amyloid plaques in (A) the cortex and (B and C) the hippocampus. (D–F) In APP/PS1;Clu−/− mice amyloid deposited predominantly in leptomeningeal and penetrating vessels in the cortex with a few parenchymal plaques observed in the hippocampus. (E and F) Arrowheads indicate Aβ deposits in small vessels in the hippocampus of APP/PS1;Clu−/−. In A–F, thioflavine-S was used to label fibrillar amyloid. (Scale bars, 100 μm.)

Fig. S2. Stereological quantification of amyloid deposition in brain parenchyma and cerebrovasculature of 6-mo-old APP/PS1 mice. (A) Six-month-old APP/PS1; Clu−/− mice showed a reduction in the amount of amyloid plaques in cortex and hippocampus. (B and C) Six-month-old mice lacking CLU had a significant increase in amyloid in (B) leptomeningeal and (C) penetrating vessels in cortex and hippocampus compared with control littermates. (D) The ratio of CAA to thioflavine-S+ plaques was significantly increased in 6-mo-old APP/PS1;Clu−/− mice. (E) Six-month-old APP/PS1;Clu−/− mice showed a decrease in the amount of total Aβ in brain parenchyma in cortex and hippocampus. (F) The ratio of thioflavine-S+ amyloid plaques to total Aβ was significantly reduced in the cortex but not in the hippocampus of 6-mo-old APP/PS1;Clu−/− mice. Thioflavine-S (thio-S) was used to visualize fibrillar amyloid. n = 11–13 mice per group. For each animal three brain sections were analyzed. Data are presented as mean ± SEM and analyzed by Mann–Whitney test; *P < 0.05, **P < 0.01, ****P < 0.0001, N.S., not significant.
Fig. S3. Sex-dependent deposition of amyloid in cortex of 6- and 12-mo-old APP/PS1 mice. (A–D) No significant difference between sexes in amyloid deposition in brain parenchyma and cerebrovasculature of 6-mo-old APP/PS1 mice was found. (B and C) The significant effect of CLU genotype on CAA was only observed for females but not males of 6-mo-old APP/PS1 mice and females had higher level of CAA compared with males in Clu<sup>−/−</sup> mice. (E) Female APP/PS1; Clu<sup>+/+</sup> mice had a significant increase in amyloid plaque pathology compared with males. No significant difference in the absence of CLU between males and females in the amount of amyloid plaques was noted. (F) No significant effect between males and females in the amount of amyloid in leptomeningeal vessel was observed. (G) Female APP/PS1; Clu<sup>−/−</sup> mice showed a significant increase in CAA in penetrating arterioles compared with males. (H) No difference between males and females in the ratio of CAA to thioflavine-S<sup>+</sup> plaque load was found. n = 5–8 mice per group. For each animal, three brain sections were analyzed. Data are presented as mean ± SEM and analyzed by Mann–Whitney test; *P < 0.05, **P < 0.01, ***P < 0.001, N.S., not significant.
Fig. S4. Sex-dependent deposition of amyloid in hippocampus of 6- and 12-mo-old APP/PS1 mice. (A and C) Six-month-old female APP/PS1;Clu−/− mice showed a significant increase in (A) the amyloid plaque load and (C) CAA in penetrating vessels compared with male APP/PS1;Clu−/− mice. (B and D) No significant difference in amyloid deposition in leptomeningeal vessels and the ratio of CAA to thioflavine-S plaque load between males and females of 6-mo-old APP/PS1 mice was observed. (E and G) Sex-dependent deposition of amyloid in the form of plaques and CAA in penetrating vessels in APP/PS1;Clu+/+ and APP/PS1;Clu−/− mice was noted. (F and H) No significant difference in the presence and absence of CLU between males and females in the amyloid deposition in leptomeningeal vessels and the ratio of CAA to parenchymal plaques was observed. n = 5–8 mice per group. For each animal, three brain sections were analyzed. Data are presented as mean ± SEM and analyzed by Mann–Whitney test; *P < 0.05; **P < 0.01; N.S., not significant.
Fig. S5. CLU expression influences the levels of soluble and insoluble Aβ in cortex and hippocampus of 6-mo-old APP/PS1 mice. (A–F) Quantification of Aβ concentrations in the cortex and hippocampus of 6-mo-old mice by ELISA. (A) APP/PS1; Clu−/− mice showed a reduction in the concentrations of insoluble Aβ40 and Aβ42 in the cortex compared with control APP/PS1; Clu+/+ mice. (B and C) APP/PS1; Clu−/− mice had also a decrease of (B) soluble and (C) detergent-soluble levels of Aβ40 and Aβ42 in cortex. (D–F) The levels of (D) insoluble and (E and F) soluble Aβ42 but not Aβ40 in hippocampus of APP/PS1; Clu−/− were also reduced. n = 15–23 mice per group. Data are presented as mean ± SEM and analyzed by Mann–Whitney test; **P < 0.01, ***P < 0.001, ****P < 0.0001, N.S., not significant.

Fig. S6. Loss of CLU decreases the total amount of neuritic dystrophy in 6-mo-old APP/PS1 mice. (A) In APP/PS1; Clu+/+ mice all parenchymal plaques (green) were surrounded by dystrophic neurites (red) identified with Lamp1 antibody. (B) APP/PS1; Clu−/− mice had an overall reduction in the amount of parenchymal plaques and a concomitant reduction in total neuritic dystrophy. Arrowheads indicate CAA. (Scale bars in A and B, 50 μm.) (C) However, prominent neuritic dystrophy was seen around parenchymal plaques that do form in APP/PS1; Clu−/− mice. (Scale bar, 20 μm) (D) Quantification of Lamp1 intensity in brain sections of 6-mo-old APP/PS1 mice. n = 8–12 mice per group. Data are presented as mean ± SEM and analyzed by Mann–Whitney test; ****P < 0.0001.
Fig. S7. CLU does not affect APP processing in APP/PS1 mice. (A) Immunoblot of cortical TBS-X brain lysates obtained from APP/PS1;Clu\(^{+/-}\) and APP/PS1;Clu\(^{-/-}\) mice showing bands corresponding to full-length APP and sAPP\(\alpha\). GAPDH was used as a control for equal loading of protein lysates. (B and C) Quantitative analysis of protein levels in the cortex. No significant differences in the level of full-length APP and sAPP\(\alpha\) were detected. Data normalized to GAPDH. \(n = 3-4\) mice per group. Data are presented as ± SEM and analyzed by Mann-Whitney test. N.S. not significant.

Table S1. Differentially expressed transcripts from RNAseq analysis of whole-brain tissue

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RNAseq analysis of Clu\(^{+/-}\) vs. Clu\(^{-/-}\) mice, expressed as reads per kilobase per million mapped reads (RPKM), identified only four differentially expressed transcripts when corrected for false-discovery rate, with minor reductions in Slc25a37 and Clu and minor increases in Hprt and Frem1.

*Nonzero because of a few reads in nontargeted exons in the Clu locus.
Table S2. RNAseq transcriptomic analysis from whole-brain tissue

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