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-β (Aβ) 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 Aβ, suggesting it might play a key role in regulating the balance between Aβ deposition and clearance in both brain and blood vessels. Here, we investigated the effect of CLU on Aβ pathology using the APP/PS1 mouse model of AD amyloidosis on a *Clu*$^{+/+}$ or *Clu*$^{-/-}$ 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*$^{-/-}$ mice. Surprisingly, despite the several-fold increase in CAA levels, APP/PS1; *Clu*$^{-/-}$ mice had significantly less hemorrhage and inflammation. Mice lacking CLU had impaired clearance of Aβ *in vivo* and exogenously added CLU significantly prevented Aβ binding to isolated vessels *ex vivo*. These findings suggest that in the absence of CLU, Aβ clearance shifts to perivascular drainage pathways resulting in fewer parenchymal plaques but more CAA due to loss of CLU chaperone activity, complicating the potential therapeutic targeting of CLU for AD.

Clusterin | Alzheimer's disease | Cerebral Amyloid Angiopathy | Aβ | hemorrhage

Introduction

Alzheimer's disease (AD) is the most common form of age-related dementia and represents a major health problem in the growing population of elderly people in developed countries (1). AD is characterized by pathological accumulation of tau as neurofibrillary tangles and deposition of toxic aggregates of amyloid-β (Aβ) peptide as fibrillar and diffuse plaques, resulting from the proteolytic cleavage of amyloid precursor protein (APP) by β- and γ-secretases (2-6). Additionally, Aβ can accumulate within the cerebral blood vessel walls, termed cerebral amyloid angiopathy (CAA). CAA is observed in the vast majority of AD patients (7-9), with Aβ deposition typically occurring in leptomeningeal vessels and penetrating arterioles (10). Several clinical complications arise from CAA, among which intracerebral hemorrhage is the most devastating (11). Additionally, familial forms of CAA arise from mutations within the Aβ coding region resulting in enhanced Aβ aggregation in the basement membrane of the cerebrovasculature (12-15).

Rare forms of AD also exist from mutations in amyloid precursor protein (APP) (16, 17) and other causative genes (18-20) leading to accelerated Aβ production and deposition, predominantly in the form of $A\beta_{42}$ (21, 22). However, it is still unclear what drives Aβ deposition in the more common sporadic form of AD. Growing evidence suggests that disruption of Aβ clearance mechanisms from the brain contributes to its accumulation, ultimately initiating the pathogenic cascade in AD (23). It has been shown that CAA can be induced by the failure of the perivascular drainage pathway to clear Aβ from the brain along cerebrovascular basement membranes (24). We have discovered several factors involved in perivascular drainage of Aβ including apolipoprotein E (ApoE), aging, and high fat diet (25-27). Therefore, uncovering additional factors that contribute to Aβ clearance by any means is critical to further our understanding of how Aβ 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 70-80kDa highly glycosylated protein that is cleaved to form α- and β 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 hu-

Significance

Deposition of amyloid-β (Aβ) peptide in the form of parenchymal plaques and Aβ 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 amyloid-β in the APP/PS1 mouse model of AD. The absence of CLU in these mice is associated with a lower number of hemorrhage and a decrease in inflammation. These results suggest that CLU functions as a major Aβ chaperone to maintain Aβ solubility along interstitial fluid drainage pathways and prevent CAA formation.

Reserved for Publication Footnotes

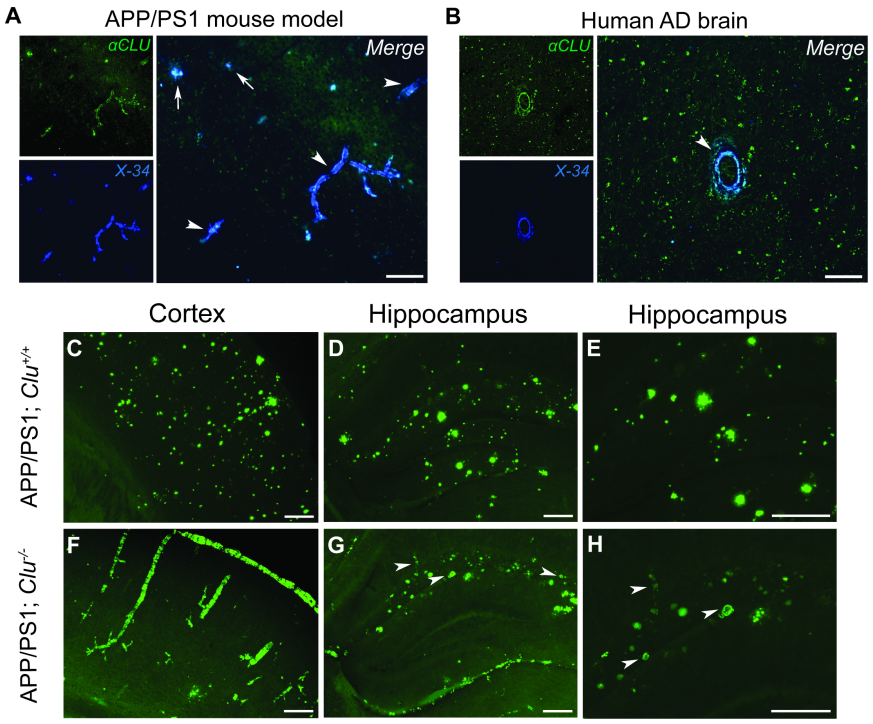

Fig. 1. CLU expression influences Aβ pathology associated with AD in cortex and hippocampus of 12-month-old APP/PS1 mice.(***A-B***) CLU-colocalization with amyloid in brain parenchyma and cerebrovasculature in the APP/PS1 mouse model and in human AD brain tissue. X-34 was used to label fibrillar amyloid. Scale bar, 50μm. (***A***) Representative brain section from APP/PS1 mouse showing halo-like co-localization of CLU (green) with amyloid plaques (blue) and complete co-localization with CAA (blue). (***B***) Representative brain section from patient with CAA showing co-localization of CLU with amyloid in cerebral vessel. Arrows indicate amyloid plaques and arrowheads indicate CAA. (***C-E***) APP/PS1; $Clu^{+/+}$ mice had abundant amyloid plaque pathology by 12 months of age in the cortex and hippocampus. (***F-H***) However, APP/PS1; $Clu^{-/-}$ mice had a striking reduction in the amount of amyloid plaques in brain parenchyma and an increase in the amount of CAA in the cortex and hippocampus. (***G-H***) Arrowheads indicate Aβ deposits in small vessels in the hippocampus of APP/PS1; $Clu^{-/-}$, rarely present in APP/PS1; $Clu^{+/+}$ mice. (***C-H***) Thioflavine-S was used to label fibrillary amyloid. Scale bar, 100μm.

man transcript. Notably, nuclear CLU has been shown to trigger apoptosis in the cells under pathological conditions (40, 41).

CLU is ubiquitously expressed in most mammalian tissues (29, 42-44), with the highest expression level in the central nervous system (CNS)(45-47). For over two decades the ε4 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 to non-demented 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 (57-59).

In addition to functional studies supporting the role of CLU in AD, genome wide association studies (GWAS) (60-63) have previously shown that genetic allelic variance in *CLU* single nucleotide polymorphisms (SNPs) are significantly associated with AD risk. More recently, rare *CLU* variants associated with AD have also been identified (64). Although a previous study utilizing 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 utilized the well-characterized APP/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 to 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 $Aβ_{40}$ and $Aβ_{42}$ 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 due to loss of CLU chaperone function. 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 co-localizes 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 co-localization 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. 1*A***). CLU also extensively co-localized to Aβ deposits in cerebral blood vessels in APP/PS1 mice (**Fig. 1*A***). In addition, CLU showed association with Aβ deposits in human cortex from an AD case with complete co-localization with CAA (**Fig. 1*B***).

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 months 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-month-old APP/PS1; $Clu^{+/+}$ mice showed Aβ deposition mostly in the form

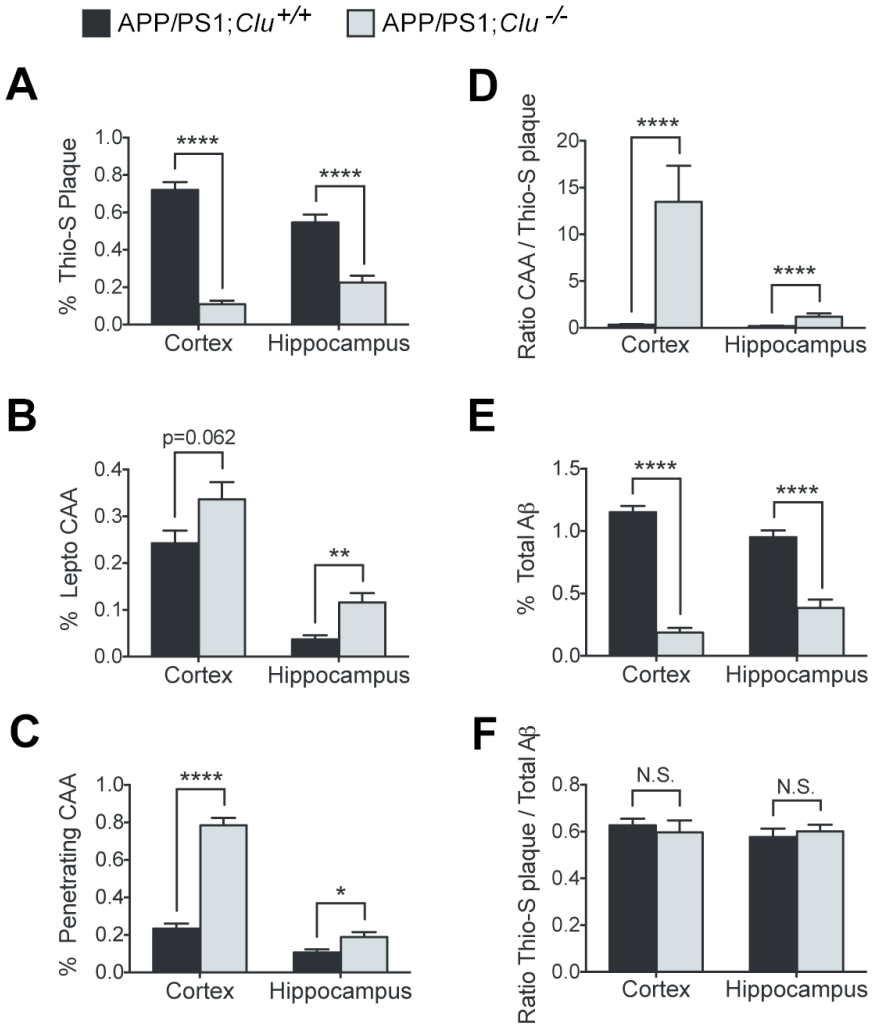

Fig. 2. Stereological quantification of amyloid deposition in brain parenchyma and cerebrovasculature in 12-month-old APP/PS1 mice. (***A***) 12-month-old APP/PS1; *Clu*$^{-/-}$ mice had a significant decrease in the amount of amyloid plaques in cortex and hippocampus.(***B***) Significant increase in 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 thio-S positive plaques was significantly increased in 12-month-old APP/PS1; *Clu*$^{-/-}$ mice. (***E***) 12-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 thio-S positive amyloid plaques to total Aβ was not different between CLU genotypes in cortex and hippocampus.Thioflavine-S (thio-S) was used to visualize fibrillar amyloid. N=11-13 mice/group. For each animal three brain sections were analyzed. Data are presented as mean ± s.e.m. and analyzed by Mann-Whitney test; *P<0.05, **P<0.01, ****P<0.0001, N.S. not significant.

of parenchymal plaques observed in the cortex (**Fig. S1A** and **Fig. 1C**) and hippocampus (**Fig. S1*B*-S1C** and **Fig. 1*D*-1*E***) whereas in APP/PS1; *Clu*$^{-/-}$ mice, Aβ was predominantly deposited in the cerebrovasculature as CAA (**Fig. S1*D*-1*F*** and **Fig. 1*F*-1*H***). To more thoroughly analyze this dramatic shift in Aβ localization, we performed an unbiased stereological quantification of thioflavine-S positive deposits in brain parenchyma and cerebrovasculature in 6- and 12-month-old mice (**Fig. S2** and **Fig. 2**). We observed a highly significant reduction in the amount of thioflavine-S positive plaques in 6-month-old APP/PS1; *Clu*$^{-/-}$ mice in cortex (P<0.0001; **Fig. S2*A***) and hippocampus (P<0.05; **Fig. S2*A***) compared to control APP/PS1; *Clu*$^{+/+}$ littermates. The absence of CLU also caused an increase in thioflavine-S positive Aβ accumulation in leptomeningeal vessels (P<0.01 in cortex and P<0.05 in hippocampus; **Fig. S2*B***) and penetrating arterioles (P<0.05 in cortex and hippocampus; **Fig. S2*C***) at 6 months of age. Similarly, 12-month-old APP/PS1; *Clu*$^{-/-}$ mice also showed reduced thioflavine-S positive deposits in parenchymal plaques (P<0.0001 in cortex and hippocampus; **Fig. 2*A***) and increased CAA in leptomeningeal vessels of cortex (P=0.062; **Fig. 2*B***) and hippocampus (P<0.01; **Fig. 2*B***) and penetrating arterioles (P<0.001 in cortex and P<0.05 in hippocampus; **Fig. 2*C***). In addition, the ratio of CAA to amyloid plaques was significantly increased in these brain regions in 12-month-old APP/PS1; *Clu*$^{-/-}$ mice compared to APP/PS1; *Clu*$^{+/+}$ mice (**Fig. 2*D***). Quantitatively, we observed a 40-fold and 6-fold increase in the ratio of CAA to parenchymal amyloid load in cortex (P<0.0001) and hippocampus (P<0.0001) of 12-month-old animals, respectively (**Fig. 2*D***). Similarly, at 6 months of age, the ratio of CAA to amyloid plaques was 50-fold increased in cortex (P<0.0001) and 5-fold increased in hippocampus (P<0.01) (**Fig. S2*D***). Numerous small vessels of the hippocampus were thioflavine-S positive in APP/PS1; *Clu*$^{-/-}$ mice (**Fig. S1*E*-1*F*** and **Fig. 1*G*-1*H***), a feature rarely seen in this APP/PS1 model.

Given that Aβ peptide accumulates in the brain in the form of fibrillar (thioflavine-S positive) and diffuse (thioflavine-S negative) plaques, we next examined the total amount of Aβ in the same animal cohort by Aβ immunostaining and stereological quantification. We observed a significant decrease in total Aβ plaque levels in 6- (P<0.0001 in cortex and P<0.05 in hippocampus; **Fig. S2*E***) and 12-month-old (P<0.0001; **Fig. 2*E***) APP/PS1; *Clu*$^{-/-}$ mice compared to APP/PS1; *Clu*$^{+/+}$ littermates. Additionally, the ratio of fibrillar plaques to total Aβ did not differ between CLU genotypes (**Fig. S2*F*** and **2*F***) with the exception of the cortical region of 6-month old animals that showed a significant reduction in this ratio in APP/PS1; *Clu*$^{-/-}$ mice in relation to APP/PS1; *Clu*$^{+/+}$ mice (P<0.0001; **Fig. S2*F***). Finally, we evaluated sex-dependent effects of CLU on amyloid pathology in 6- and 12-month old-mice (**Fig. S3** and **Fig. S4**). We observed significant differences in amyloid plaque formation (**Fig. S3*E***, **Fig. S4*A*** and **S4*E***) and CAA in penetrating vessels (**Fig.S3*G***,

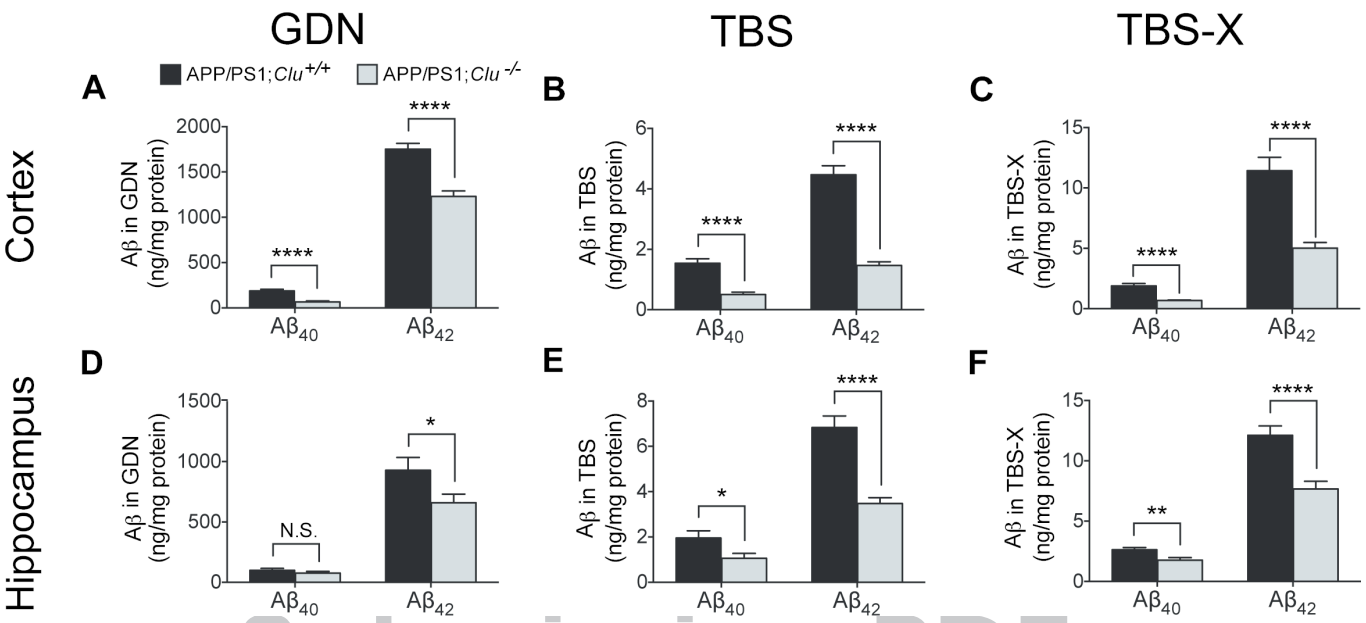

Fig. 3. CLU expression alters the levels of soluble and insoluble Aβ in cortex and hippocampus of 12-month-old APP/PS1 mice. (***A-F***)Quantification of the Aβ levels in cortex and hippocampus of 12-month old APP/PS1 mice by ELISA. (***A***)APP/PS1; *Clu*$^{-/-}$ mice showed a significant decrease in the levels of insoluble $A\beta_{40}$ and$A\beta_{42}$ in cortex compared to control APP/PS1; *Clu*$^{+/+}$. (***B***, ***C***) APP/PS1; *Clu*$^{-/-}$ mice had also reduced levels of(***B***)soluble and(***C***)detergent-soluble concentrations of $A\beta_{40}$ and $A\beta_{42}$ incortex.(***D***)The levels of insoluble$A\beta_{42}$ but not$A\beta_{40}$ in hippocampus of APP/PS1; *Clu*$^{-/-}$ mice were also reduced.(***E, F***)Concentration of(***E***)soluble and (***F***)detergent-soluble levels of$A\beta_{40}$ and$A\beta_{42}$ in hippocampus were decreased in the absence of CLU. N= 15-23 mice/group. Data arepresented as mean ± s.e.m. and analyzed by Mann-Whitney test. *P<0.05, **P<0.01, ****P<0.0001, N.S. not significant.

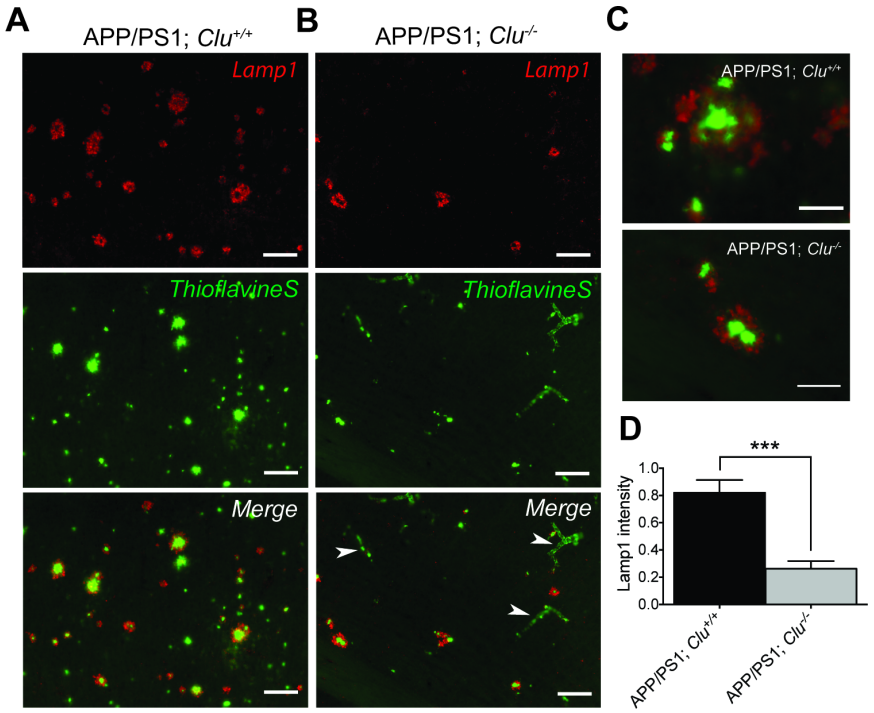

Fig. 4. Absence of CLU reduces the total amount of neuritic dystrophy in APP/PS1 mice. (***A***)In APP/PS1; *Clu*$^{+/+}$ mice all parenchymal plaques (green) were surrounded by dystrophic neurites (red) identified with Lamp1antibody. (***B***)APP/PS1; *Clu*$^{-/-}$ mice had an overall reduction in the amount of parenchymal plaques and a concomitant reduction in total neuritic dystrophy. Arrowheads indicate CAA. (***A-B***) Scale bar, 50μm. (***C***)However, prominent neuritic dystrophy was seen around parenchymal plaques that do form in APP/PS1; *Clu*$^{-/-}$ mice. Scale bar, 20μm.(***D***)Quantification of Lamp1 intensity in brain sections of12-month-old APP/PS1 mice. N=8-12 mice/group. Data are presented as mean ± s.e.m. and analyzed by Mann-Whitney test. ***P<0.001.

Fig. S4*C* and **S4*G***) in 6- and 12-month-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

Since CLU expression significantly impacts where Aβ deposits in the brain, we next examined whether CLU genotype alters the levels of extractable forms of Aβ. Enzyme-linked immunosorbent assay (ELISA) was used to analyze insoluble (guanidine-HCl fraction, GDN) as well as TBS-soluble and detergent soluble (TBS with Trition-X-100, TBSX) forms of $A\beta_{40}$ and $A\beta_{42}$ from cortex and hippocampus of 6- and 12-month-old APP/PS1; *Clu*$^{+/+}$ and APP/PS1; *Clu*$^{-/-}$ mice (**Fig. S5** and **Fig. 3**). In both APP/PS1; *Clu*$^{+/+}$ and APP/PS1; *Clu*$^{-/-}$ mice, substantially higher concentrations of $A\beta_{40}$ and $A\beta_{42}$ were found in the insoluble fraction relative to soluble Aβ forms within each genotype (**Fig. S5** and **Fig. 3**), reflecting that the majority of

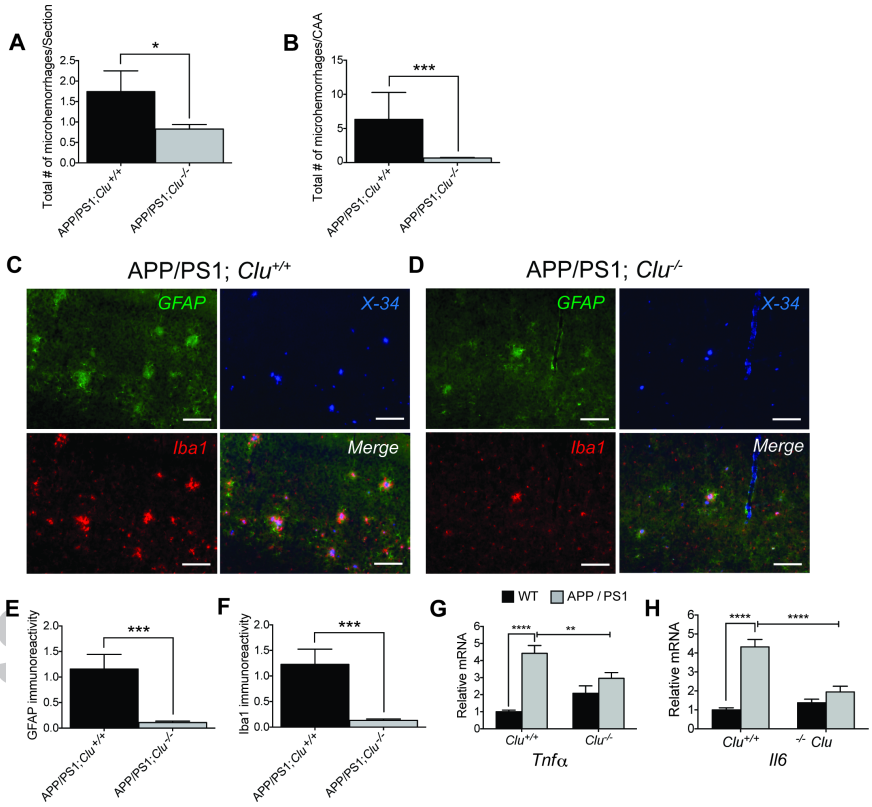

Fig. 5. The absence of CLU in APP/PS1 mice prevents hemorrhage and inflammation at the cellular and molecular level. (***A-B***) Quantification of CAA-associated hemorrhage in 12-month-old APP/PS1 mice. (***A***) Despite the abundant increase in CAA, APP/PS1; *Clu*$^{-/-}$ mice had significantly reduced total number of microhemorrhages and (***B***) number of microhemorrhages normalized to CAA load compared to control APP/PS1; *Clu*$^{+/+}$ animals. (***C***) APP/PS1; *Clu*$^{+/+}$ mice had abundant astrogliosis (green) and microgliosis (red) surrounding amyloid plaques (blue). (***D***) Despite the dramatic increase in CAA in APP/PS1; *Clu*$^{-/-}$ mice, the overall level of gliosis was significantly reduced. (***C-D***) Scale bar, 50μm. (***E***) Quantification of astrogliosis showed the significant decrease in the number of reactive astrocytes in APP/PS1; *Clu*$^{-/-}$ mice compared to control. (***F***) Quantification of microgliosis showing reduction of reactive microglia in APP/PS1; *Clu*$^{-/-}$ mice compared to APP/PS1; *Clu*$^{+/+}$ animals. (***G-H***) APP/PS1; *Clu*$^{+/+}$ mice had increased levels of proinflammatory cytokines such as *Tnfα* and *Il6* compared to non-transgenic (nTg) *Clu*$^{+/+}$ animals. However, this increase in proinflammatory cytokines was significantly reduced in APP/PS1; *Clu*$^{-/-}$ mice. (***A-B***) N=11-13 mice/group. For each animal 18-21 brain sections were analyzed. Data are presented as mean ± s.e.m. and analyzed by Student's t-test; * P<0.05 and *** P<0.001. (***E-F***) N=7-11 mice/group. Data are presented as mean ± s.e.m. and analyzed by Student's t-test ***P<0.001. (***G-H***) N=8-10 mice/group. Data are presented as mean ± s.e.m. and analyzed by two-way ANOVA with post-hoc Tukey's test. **P<0.01, ****P<0.0001.

Aβ is deposited as insoluble parenchymal plaques and CAA, respectively. Relative to controls, APP/PS1*; Clu*$^{-/-}$ mice showed significantly lower levels of $A\beta_{40}$ and $A\beta_{42}$ in the GDN fraction from cortex at 6 and 12 months of age (P<0.0001; **Fig. S5*A*** and **3*A***) and $A\beta_{42}$ from hippocampus at 6 and 12 months of age (P<0.05; **Fig. S5*D*** and **3*D***). Hippocampal levels of insoluble $A\beta_{40}$ were not statistically different between CLU genotypes (**Fig. S5*D*** and **3*D***). Similarly, TBS and TBS-X soluble fractions showed dramatic reduction of $A\beta_{40}$ and $A\beta_{42}$ levels in the cortex of 6- (P<0.01 and P<0.0001; **Fig. S5*B*** and P<0.0001; **Fig. S5*C***) and 12-month old (P<0.0001; **Fig. 3*B*** and P<0.0001; **3*C***) APP/PS1*; Clu*$^{-/-}$ mice in relation to control APP/PS1; *Clu*$^{+/+}$ mice. Additionally, we found that hippocampal concentrations of soluble $A\beta_{40}$ and $A\beta_{42}$ of 12-month old mice APP/PS1*; Clu*$^{-/-}$ (P<0.05, P<0.0001; **Fig. 3*E*** and P<0.01, P<0.0001; **3*F***) and $A\beta_{42}$ of 6-month old mice APP/PS1*; Clu*$^{-/-}$ (P<0.001; **Fig. S5*E*** and P<0.01; **Fig. S5*F***) were significantly decreased relative to APP/PS1; *Clu*$^{+/+}$ controls. These data indicate that CLU expression alters the biochemical levels of Aβ deposition and is in agreement with the histological results.

Loss of CLU significantly reduces parenchymal plaque load and neuritic dystrophy

Previous studies utilizing 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 CLU genotype affects neuritic dystrophy, we performed double labeling of brain sections with Lamp1, to mark dystrophic neurites, and thioflavine-S, to define fibrillar plaques (**Fig. S6*A-C*** and **Fig. 4*A-C***). As expected, we found numerous dystrophic neurites around parenchymal plaques in 6- (**Fig. S6*A***) and 12-month-old APP/PS1; *Clu*$^{+/+}$ mice (**Fig. 4*A***) but none observed in proximity to CAA alone (**Fig. S6*B*** and **Fig. 4*B***). 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 to APP/PS1; *Clu*$^{+/+}$ mice (**Fig. S6*B***, **S6*D*** and **Fig. 4*B*, 4*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 positive plaques that were detected in APP/PS1; *Clu*$^{-/-}$ mice (**Fig. S6*C*** and **Fig. 4*C***). The discrepancy between our results and previous reports may be due to differences in the APP transgenic model or the mixed genetic background of Demattos et al., 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

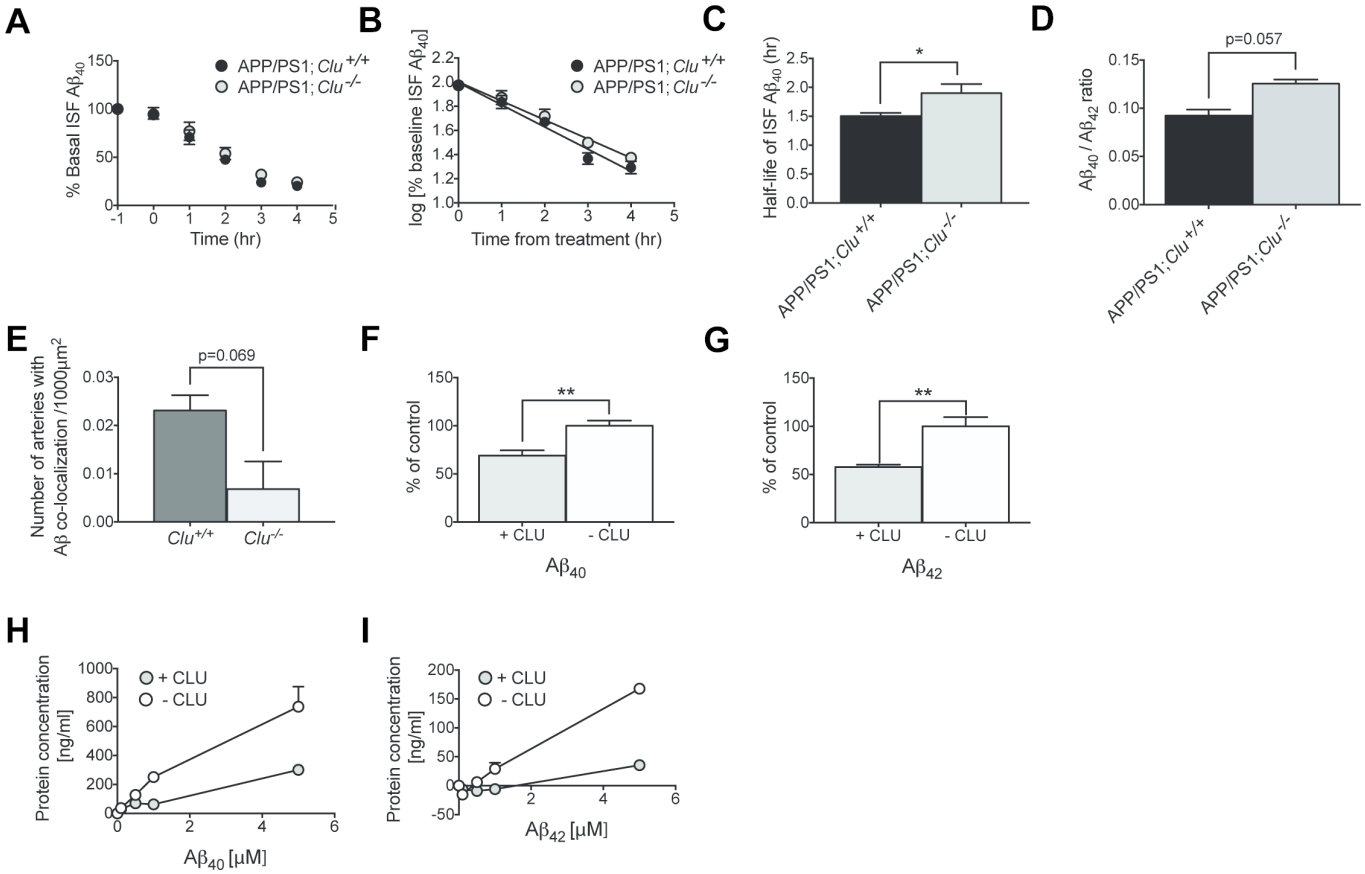

Fig. 6. CLU alters Aβ clearance and prevents binding of soluble Aβ to cerebrovasculature. (***A-C***) *In vivo* microdialysis to assess the Aβ metabolism in the hippocampus of 10-week-old APP/PS1; *Clu*$^{+/+}$ and APP/PS1; *Clu*$^{-/-}$. (***A***)The concentration of $A\beta_{40}$ in ISF was measured as the basal level of Aβ. (***B***) The logarithm (log) of percentage baseline ISF $A\beta_{40}$ versus time was plotted after treatment of mice with a potent γ-secretase inhibitor. (***C***) The slope from the linear regressions from log (% ISF Aβ40) was used to asses the half-life of $A\beta_{40}$ elimination from the ISF. (***D***) The ratio of Aβ*40:42* was examined from 10-week-old APP/PS1; *Clu*$^{+/+}$ and APP/PS1; *Clu*$^{-/-}$. (***E***) Quantification of the number of arteries with co-localization of fluorescently labeled human $A\beta_{40}$. Fewer arteries with Aβ co-localization were observed in *Clu*$^{-/-}$mice compared to *Clu*$^{+/+}$ animals. (***F-G***) Exogenous (***F***) $A\beta_{40}$ and (***G***) $A\beta_{42}$ were applied to isolated cerebral vessels with and without exogenously added CLU and binding of Aβ was measured by ELISA. The addition of 1μM exogenous CLU led to a significant reduction in the levels of (***F***) $A\beta_{40}$ and (***G***) $A\beta_{42}$ associated with cerebrovasculature. (***H-I***) Isolated vessels were treated with increasing concentrations of exogenous (***H***) $A\beta_{40}$ and (***I***) $A\beta_{42}$ in the absence and presence of increasing concentrations of CLU. CLU addition resulted in a decrease amount of (***H***) $A\beta_{40}$ and (***I***) $A\beta_{42}$ bound to cerebral vessels even at high Aβ concentrations. Data presented as ELISA replicates. (***A-C***) N= 5-6 mice/group. Data are presented as ± s.e.m and analyzed by Student's t-test. *P<0.05. (***E-I***) N= 3-4 mice/group. Data are presented as ± s.e.m and analyzed by Student's t-test. **P<0.01.

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-month-old APP/PS1; *Clu*$^{+/+}$ and APP/PS1; *Clu*$^{-/-}$ mice (N≥18 sections/mouse spaced 300μ apart). APP/PS1 mice predominantly develop microhemorrhages in cortex and hippocampus since 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 to control APP/PS1; *Clu*$^{+/+}$ mice (P<0.05; **Fig. 5*A***). 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. 5*B***).

Given that both parenchymal plaques and CAA are independently associated with neuroinflammation (67), we next investigated whether CLU genotype had a differential effect on gliosis. Abundant astrogliosis was present around amyloid plaques in brain parenchyma of APP/PS1; *Clu*$^{+/+}$ mice (**Fig. 5*C***). Although the absence of CLU resulted in a dramatic increase in CAA, the level of astrogliosis was significantly reduced when assessed at 12 months of age in APP/PS1; *Clu*$^{-/-}$ mice (**Fig. 5*D*** and **5*E***). Similarly, APP/PS1; *Clu*$^{-/-}$ mice had significantly decreased microgliosis compared to APP/PS1; *Clu*$^{+/+}$ mice (**Fig. 5*D*** and **5*F***). Reactive astrocytes and microglia were not observed in proximity to CAA regardless of *Clu* genotype (**Fig. 5*D***). To test whether *Clu* genotype also affected neuroinflammation at the molecular level, we profiled inflammatory cytokine transcripts, tumor necrosis factor alpha (*Tnf*α) and interleukin 6 (*Il6*), by real-time quantitative PCR (RT-qPCR). The levels of *Tnf*α and *Il6* were significantly increased in APP/PS1; *Clu*$^{+/+}$ compared to non-transgenic *Clu*$^{+/+}$ littermates but these levels were significantly reduced to near baseline (non-transgenic levels) in APP/PS1; *Clu*$^{-/-}$ mice (**Fig. 5*G*-5*H***). Taken together, these experiments demonstrate that the majority of inflammation in the presence of amyloid is due to 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 determine 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.**

S7*A*). CLU genotype did not alter APP and sAPPα expression levels indicating that CLU does not grossly affect APP processing (**Fig. S7*B*** and **S7*C***).

A myriad of other factors besides APP processing could explain the shift in Aβ pathology from parenchymal plaques to CAA. 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-month-old *Clu*$^{+/+}$ and *Clu*$^{-/-}$ mice (n=4/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, *Slc25a37*, *Hprt*, and *Frem1* (**Table S1**). No significant changes were found in other AD genes such as *Apoe*, *Bin1*, *Abca7*, *Picalm*, *Cd33*, *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 parenchyma 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 utilized *in vivo* microdialysis (**Fig. 6*A*-*C***). Since soluble Aβ in the interstitial fluid (ISF) has been shown to correlate with Aβ deposited in the brain parenchyma (69) we measured the hippocampal steady state levels in 10-week-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 ($t_{1/2}$) of $Aβ_{40}$. The concentration of hippocampal $Aβ_{40}$, measured in ISF, gradually decreased over time with APP/PS1; *Clu*$^{+/+}$ showing faster decline compared to APP/PS1; *Clu*$^{-/-}$ mice (**Fig. 6*B***). Moreover the $t_{1/2}$ of ISF $Aβ_{40}$ was significantly longer in mice lacking CLU compared to control littermates (**Fig. 6*C***). These results suggest that the loss of CLU may alter the clearance of soluble Aβ from the ISF.

Numerous studies have previously shown that $Aβ_{40}$ is predominantly present in vascular amyloid due to its more soluble nature (70, 71), whereas $Aβ_{42}$, a more fibrillogenic form of Aβ, is mainly found in parenchymal amyloid (72, 73). Therefore the ratio of Aβ*40:42* seems to determine where Aβ deposits in the brain with higher Aβ*40:42* ratio predisposing the formation of CAA (74). In agreement with this hypothesis, we found a slight increase in the Aβ*40:42* ratio (p=0.057; **Fig. 6*D***) in APP/PS1; *Clu*$^{-/-}$ mice compared to APP/PS1; *Clu*$^{+/+}$ mice.

Given that CAA and AD appear to result from a disruption of perivascular drainage pathway (24, 75), we sought to investigate the potential role of CLU in the Aβ removal along the basement membrane of cerebral vessels. Therefore, we examined the pattern of distribution of fluorescently labeled human $Aβ_{40}$ following its intracerebral injection of 3-month old *Clu*$^{+/+}$ and *Clu*$^{-/-}$ mice as we have previously described (25, 26). The difference in counts of arteries with Aβ co-localization between the injection site and 100μm posterior was calculated as a measure of perivascular drainage 10 minutes after injection of fluorescently labeled $Aβ_{40}$. We detected fewer arteries with fluorescent Aβ localization in *Clu*$^{-/-}$ mice compared to control littermates (p=0.069; **Fig. 6*E***) suggesting that perivascular drainage of Aβ might be compromised in the brains of *Clu*$^{-/-}$ animals.

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* binding 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 $Aβ_{40}$ or $Aβ_{42}$ in the presence or absence of exogenous CLU and then washed, lysed in GDN buffer, and Aβ levels were assessed by ELISA assay. We found that addition of exogenous CLU resulted in a significant reduction of the amount of $Aβ_{40}$ (P<0.01; **Fig. 6*F***) and $Aβ_{42}$ (P<0.01; **Fig. 6*G***) bound to isolated cerebral blood vessels compared to samples lacking exogenous CLU. A similar effect was observed when isolated cerebral vessels were treated with increasing concentrations of exogenous $Aβ_{40}$ or $Aβ_{42}$ in the presence of equally increasing concentrations of exogenous CLU (**Fig. 6*H*** and **6*I***). The addition of exogenous CLU led to a dramatic decrease of the level of Aβ associated with the cerebrovasculature compared to 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 resulting in the aggregation and deposition in the cerebral blood vessels due to 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 parenchmal amyloid plaque accumulation and minimal CAA observed in APP/PS1; *Clu*$^{+/+}$ mice, APP/PS1; *Clu*$^{-/-}$ 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 to APP/PS1; *Clu*$^{+/+}$ mice. Importantly, by using *in vivo* microdialysis, we provided evidence that CLU is involved in the elimination of Aβ from the brain. Consistent with this notion, intracerebral injections of $Aβ_{40}$ of young *Clu*$^{+/+}$ and *Clu*$^{-/-}$ mice resulted in a decreased number of arteries with fluorescently labeled $Aβ_{40}$ implying the disruption of perivascular drainage pathway in the absence of CLU. Finally, we identified that the presence of exogenously added CLU reduced binding of $Aβ_{40}$ and $Aβ_{42}$ to isolated cerebral vessels, suggesting that CLU impacts Aβ pathology in vessels by preventing it from binding and aggregating during ISF drainage.

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

One of the most striking phenotypes of CLU loss in our AD amyloidosis model was the shift in the localization of Aβ deposition from parenchymal plaques to CAA. Although DeMattos et al. 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) and/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 CLU 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 Aβ*40:42* ratio in APP/PS1; *Clu*$^{-/-}$ mice, possibly contributing to the shift of Aβ deposition between brain compartments. Given that $A\beta_{40}$ appears to mediate accumulation of amyloid in cerebral vessels (70, 71), whereas $A\beta_{42}$ is thought to be a predominant form present in the brain parenchyma (72, 73), Aβ*40:42* ratio might be an important factor in determining where Aβ deposits.

In fact, several lines of evidence have previously suggested that a high Aβ*40:42* ratio favors the development of CAA (74, 77). The APPDutch animal model, that 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 when compared to 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 Aβ*40:42* in these animals in relation to animals expressing endogenous murine ApoE (74). In contrast, a lower Aβ*40:42* 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 $A\beta_{42}$, have a reduction in Aβ*40:42* 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 $A\beta_{42}$, 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 co-localization of injected fluorescent Aβ in the basement membranes in *Clu*$^{-/-}$ mice compared to control littermates, suggesting the disruption of perivascular drainage of Aβ in the absence of CLU. Consistent with this notion, we found direct *ex vivo* evidence that CLU alters Aβ binding to isolated cerebral vessels, which might exacerbate development of CAA. Therefore we propose that CLU 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 CLU 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 CLU protein is significantly elevated in human leptomeningeal arteries with CAA (85), suggesting the entrapment of the Aβ–CLU complex in the perivascular drainage pathways, or a compensatory up-regulation of CLU to clear Aβ.

Despite the evidence that loss of CLU 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 blood brain barrier (BBB) can be facilitated via low-density lipoprotein receptor-related protein-1 (LRP1) (84). In addition, the low density lipoprotein receptor-related protein-2 (LRP2) has been previously shown to mediate the elimination of $A\beta_{42}$ from the brain. LRP2 is a receptor for CLU localized at the BBB and it has been suggested to be essential for the transport of the Aβ-CLU complex into circulation (57). It is possible, that the absence of CLU also disrupts the Aβ transport across BBB via LRP2, leading to the accumulation of Aβ within the walls of the cerebrovasuclature. 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 *Lrp1, Lrp2,* 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. Recurrent 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) that 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 to APP/PS1; *Clu*$^{+/+}$ animals. A possible explanation for this difference with previous studies could be that CLU expression alters the structure and/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). While 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 novel 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 unin-

tentionally 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 APPswe/PS1ΔE9 were used (65). All studies were done in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals under an approved protocol from the Mayo Clinic Institutional Animal Care and Use Committee. De-identified post-mortem, pathologically confirmed Alzheimer's disease brain tissue was 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 APP/PS1; *Clu*$^{+/+}$ and APP/PS1; *Clu*$^{-/-}$ mice were used and analyzed using a Zeiss AxioImager.Z1/ApoTome microscope. Aβ pathology was quantified, as previously described(76).

Biochemical analyses

Cortex and hippocampus were dissected from APP/PS1; *Clu*$^{+/+}$ and APP/PS1; *Clu*$^{-/-}$ PBS perfused brains. Separate extraction for each condition was used. $A\beta_{40}$ and $A\beta_{42}$ levels were assessed by ELISAs. To examine APP processing, cortex of APP/PS1; *Clu*$^{+/+}$ and APP/PS1; *Clu*$^{-/-}$ mice was used. Total RNA was isolated using a Total Aurum RNA isolation kit. Random-primed 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 APP/PS1; *Clu*$^{+/+}$ and APP/PS1; *Clu*$^{-/-}$ mice was performed, as described (69, 94). Perivascular drainage was quantified in *Clu*$^{+/+}$ and *Clu*$^{-/-}$ mice, as described (25).

Aβ binding to cerebrovasculature

Cerebral vessels were isolated from C57Bl/6J mice, as described (76). Vessels were treated with $A\beta_{40}$ or $A\beta_{42}$ 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*.

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