Intracellular Clusterin Interacts with Brain Isoforms of the Bridging Integrator 1 and with the Microtubule-Associated Protein Tau in Alzheimer’s Disease

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Abstract

Sporadic or late-onset Alzheimer's disease (AD) is expected to affect 50% of individuals reaching 85 years of age. The most significant genetic risk factor for late-onset AD is the ε4 allele of APOE gene encoding apolipoprotein E, a lipid carrier shown to modulate brain amyloid burden. Recent genome-wide association studies have uncovered additional single nucleotide polymorphisms (SNPs) linked to AD susceptibility, including those in the CLU and BIN1 genes encoding for clusterin (CLU) and the bridging integrator 1 (BIN1) proteins, respectively. Because CLU has been implicated in brain amyloid-β (Aβ) clearance in mouse models of amyloid deposition, we sought to investigate whether an AD-linked SNP in the CLU gene altered Aβ42 biomarker levels in the cerebrospinal fluid (CSF). Instead, we found that the CLU rs1136000 SNP modified CSF levels of the microtubule-associated protein Tau in AD patients. We also found that an intracellular form of CLU (iCLU) was upregulated in the brain of Tau overexpressing Tg4510 mice, but not in Tg2576 amyloid mouse model. By overexpressing iCLU and Tau in cell culture systems we discovered that iCLU was a Tau-interacting protein and that iCLU associated with brain-specific isoforms of BIN1, also recently identified as a Tau-binding protein. Through expression analysis of CLU and BIN1 variants, we found that CLU and BIN1 interacted via their coiled-coil motifs. In co-immunoprecipitation studies using human brain tissue, we showed that iCLU and the major BIN1 isoform expressed in neurons were associated with modified Tau species found in AD. Finally, we showed that expression of certain coding CLU variants linked to AD risk led to increased levels of iCLU. Together, our findings suggest that iCLU and BIN1 interaction might impact Tau function in neurons and uncover potential new mechanisms underlying the etiology of Tau pathology in AD.

Introduction

Multiple genome-wide association studies (GWAS) have replicated a link between common single nucleotide polymorphisms in the CLU gene (rs1136000 and rs1532278) and increased susceptibility for late-onset Alzheimer’s disease (AD) [1–7]. In addition, rare CLU variants revealed by next-generation sequencing have also been associated with AD risk [8]. However, the mechanisms by which modifications in clusterin expression and/or function alter disease risk are not yet clear [9]. Clusterin (CLU) is synthesized as a 60–80 kD precursor protein that undergoes internal cleavage generating α- and β-chains joined by disulfide bonds [10]. This glycosylated heterodimeric CLU is constitutively secreted and referred to as soluble clusterin (sCLU), or as apolipoprotein J (apoJ), when found in association with lipoproteins [11]. Shorter forms of the precursor CLU have been detected intracellularly and named cytosolic, truncated or nuclear CLU [12–14]. Alternative splicing, internal translation initiation, mistranslocation of sCLU, and impaired proteasomal degradation all appear to contribute to the pool of cytosolic CLU isoforms [14]. The function of intracellular CLU (iCLU) is not completely understood. Studies in cancer biology have linked iCLU to Bax-mediated apoptosis [15,16]. Of relevance to AD, it has been recently shown that iCLU levels increase quickly in cultured primary neurons exposed to amyloid-β peptides (Aβ), and that this iCLU elevation is required for the neurotoxic downstream signaling effects of Aβ [17].

CLU expression is highest in the brain and is markedly upregulated under situations of stress and inflammation [18,19]. Induction of CLU mRNA is observed within pyramidal neurons of the hippocampus and the entorhinal cortex of AD patients [20,21], and CLU immunoreactivity is found in association with neurofibrillary tangles and amyloid plaques [20]. Published literature suggests that CLU plays a chaperone role for Aβ, modulating both its clearance and deposition [21–23], similar to a function proposed for apoE [24]. Seminal support for this putative role of CLU was revealed by the double deletion of
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CLU and apoE in a mouse model of amyloid deposition [23]. While no substantial changes in brain amyloid were detected with the individual deletions, double knockout (KO) mice showed a dramatic exacerbation of amyloid burden. The hypothesis is further substantiated by findings showing direct interaction between CLU and Aβ [25,26]. However, despite these suggestive preclinical findings, clinical data is lacking to corroborate a significant effect of CLU on Aβ burden as a major mechanism underlying the genetic link to AD [27,28]. Healthy carriers of the CLU rs11136000 risk allele C show decreased white matter integrity [29], altered coupling between hippocampus and prefrontal cortex during memory processing [30], and significant longitudinal increases of cerebral blood flow in the hippocampus and anterior cingulate cortex [31], indicating that CLU may also participate in non-Aβ pathways that could modulate vulnerability to AD.

First identified as a tumor suppressor [32], the bridging integrator 1 (BIN1) has been recently linked to AD susceptibility by GWAS [33–37]. BIN1 is highly expressed in the brain and all seven brain-specific BIN1 isoforms have an inserted domain that interacts with clathrin and AP2/α-adaptin (CLAP), indicating a key role for neuronal BIN1 in endocytosis [38]. The exact mechanism by which polymorphisms in the BIN1 gene alter AD risk is still unknown, but a recent study provided evidence that BIN1 interacts with the microtubule-associated protein Tau [39]. BIN1 immunoreactivity was found in co-localization with neurofibrillary tangles in the AD brain, and knockdown of the BIN1 ortholog Amph partially restored the rough eye phenotype associated with human Tau overexpression in Drosophila [39].

Here we provide evidence that the CLU rs11136000 SNP alters CSF Tau levels in AD, and that iCLU is a Tau-interacting protein elevated in the brain of Tau-overexpressing Tg4510 mice. We also demonstrate that iCLU interacts with brain-specific BIN1 isoforms containing a putative coiled-coil motif. Furthermore, an AD-risk iCLU mutant lacking the C-terminus coiled-coil motif does not interact with BIN1, suggesting that CLU and BIN1 association is mediated via their coiled-coil domains. Lastly, we show that iCLU and BIN1 isoform 1 are associated with modified Tau species found in the brain of AD patients, and that certain coding CLU mutations linked to AD risk increase the ratio between iCLU and sCLU when transfected in cells. Together, these genetic and functional studies establish a novel link between iCLU and sCLU protein elevated in the brain of Tau-overexpressing Tg4510 mice. To further substantiate our hypothesis for a potential physiologically relevant connection between iCLU and Tau, we found that a truncated iCLU form (~45–50 kD) was significantly elevated in the hippocampus of both 2-month-old pre-tangle and 5.5 month-old tangle-bearing Tg4510 in comparison to age-matched wild type (WT) mice (Fig. 2a). Unlike the clear changes in brain CLU observed with Tau overexpression, Western blot evaluation of hippocampus from Tg2576 amyloid mouse model showed no age-dependent changes in either forms of CLU compared to WT littermate controls (Fig. 2b). These findings further substantiated our hypothesis for a potential physiologically relevant connection between iCLU and Tau.

### Results

CLU rs11136000 alters CSF Tau in AD patients

To assess potential mechanisms by which CLU confers risk for AD, we examined effects of the CLU rs11136000 SNP on CSF Aβ42 and Tau levels, which are reliable disease biomarkers recently used as endophenotypes in AD genetic studies [40]. For this analysis, the AD population was enriched with patients presenting a disease CSF Tau/Aβ42 profile, which has a high diagnostic accuracy for AD over other types of dementia [41,42]. The CSF from an initial set of 80 patients diagnosed with probable AD and 50 control non-demented individuals was evaluated for Aβ42 and Tau content by specific ELISAs. Using receiving operating curve (ROC) analysis of CSF Tau/Aβ42 ratio to establish a cut-off value (Fig. 1a), we identified 25 clinically diagnosed demented individuals who lacked the projected CSF AD profile, and were therefore subtracted from experimental patient population (Fig. 1b). Because of previously reported effects of apoE genotype on brain amyloid burden [24], we also characterized subjects for their APOE variants. When stratified by rs11136000 and APOE genotypes, we found a significant effect of the risk allele C on CSF Tau levels in AD patients carrying apoE4, with homozygous CC individuals showing significantly higher CSF Tau than heterozygous CT carriers (Fig. 1c). Consistent with previous reports [43,44], no significant CLU genotype effects were observed on CSF Aβ42 levels within AD patients. In addition, CSF AD biomarkers Aβ42 and Tau were not affected by the CLU genotypes in either apoE4 or non-apoE4 control subjects (Fig. 1c). While CSF CLU protein levels were not impacted by CLU genotypes, Tau/Aβ42 confirmed AD patients had significantly higher CSF CLU than non-demented controls (Fig. 1d), consistent with previous literature findings [45,46]. Based on these initial results, we hypothesized a potential link between CLU and Tau pathology in AD.

CLU is upregulated in the brain of Tg4510 Tau mouse model

We next evaluated the expression pattern of CLU isoforms in the hippocampus and cortex of Tg4510 mice overexpressing the human mutant P301L Tau associated with frontotemporal dementia [47]. While 5.5 month-old Tg4510 mice displaying Tau pathology and neurodegeneration showed marked upregulation of sCLU protein (~30–40 kD monomer) in the hippocampus, we found that a truncated iCLU form (~45–50 kD) was significantly elevated in the hippocampus of both 2-month-old pre-tangle and 5.5 month-old tangle-bearing Tg4510 in comparison to age-matched wild type (WT) mice (Fig. 2a). Unlike the clear changes in brain CLU observed with Tau overexpression, Western blot evaluation of hippocampus from Tg2576 amyloid mouse model showed no age-dependent changes in either forms of CLU compared to WT littermate controls (Fig. 2b). These findings further substantiated our hypothesis for a potential physiologically relevant connection between iCLU and Tau.

iCLU is localized to the cytoskeleton fraction and interacts with Tau

Because iCLU had been previously detected in both cytosol and nucleus, we first examined the subcellular fractionation of CLU following transfection of full-length or truncated CLU cDNA constructs in HEK 293T cells (Fig. 3a). Interestingly, we found that most of the ~52 kD iCLU was detected in the cytoskeleton/insoluble fraction. The precursor CLU protein (~64 kD) was fractionated primarily to the cytosol, as predicted by ER/Golgi localization. While the majority of sCLU (~37 kD) was localized to the membrane fraction, likely associated with secretory Golgi vesicles, some sCLU was also detected in the cytosolic fraction. The primary localization of overexpressed iCLU to the cytoskeleton fraction led us to postulate the possibility of a direct interaction between iCLU and the microtubule-associated protein Tau.

To further investigate a potential interaction between CLU and Tau, we used a doxycycline (dox)-inducible wild-type Tau HEK 293T (iTau-HEK) cell line transfected with either full-length or truncated CLU cDNA constructs. By overexpressing the truncated CLU construct designed to generate only the iCLU 50 kD form, we found that the iCLU construct designed to generate only the iCLU 50 kD form partially restored the rough eye phenotype in binauric mice [25,26]. However, despite these suggestive preclinical findings, clinical data is lacking to corroborate a significant effect of CLU on Aβ burden as a major mechanism underlying the genetic link to AD [27,28]. Healthy carriers of the CLU rs11136000 risk allele C show decreased white matter integrity [29], altered coupling between hippocampus and prefrontal cortex during memory processing [30], and significant longitudinal increases of cerebral blood flow in the hippocampus and anterior cingulate cortex [31], indicating that CLU may also participate in non-Aβ pathways that could modulate vulnerability to AD.
brain could interact with iCLU. Co-transfection of Flag-tagged iCLU and WT or P301L mutant 4-repeat full-length (4R2N) Tau into HEK 293T cells followed by pull-down with anti-Tau antibody revealed that both WT and mutant P301L Tau interacted similarly with iCLU (Fig. 3c). Reversely, pull-down of iCLU with anti-Flag antibody co-immunoprecipitated both WT and P301L full-length Tau, but not truncated Tau forms containing only the 4-repeat microtubule binding domain (MBD) (Fig. 3c). Together, these cellular studies indicated that iCLU localizes primarily to the cytoskeleton fraction of cellular extracts and interacts with both WT and P301L mutant full-length Tau. The iCLU-Tau interaction was detected in both Tau-inducible stable cell lines and in transient Tau transfection experiments, and was confirmed by reciprocal co-immunoprecipitation. In addition, our results also showed that the 4-repeat MBD of Tau alone did not show interaction with iCLU.

iCLU interacts with brain-specific isoforms of BIN1 containing a coiled-coil motif

It has been recently shown that BIN1, a BAR-protein highly expressed in the brain and recently linked to AD risk, is a Tau-interacting protein [39]. Ten isoforms of human BIN1 are produced by alternative splicing of the BIN1 gene [38]. Isoforms 1–7 are brain-specific and contain a CLAP domain involved in endocytosis. In addition, brain isoforms 1–3 contain a 31 amino acid insert within the BAR domain coding a putative coiled-coil region [38]. CLU has 2 coiled-coil motifs and has been reported to interact with other coiled-coil containing proteins, including the apoptosis-related Ku70 [48] and the microtubule-destabilizing neuronal protein SCLIP (SCG10-like protein) [49]. In light of this evidence, we sought to investigate whether iCLU interacted with coiled-coil containing BIN1 isoforms, and if BIN1 association with Tau was altered upon overexpression of iCLU. Following co-transfection of iCLU with each one of the ten myc-tagged BIN1 isoforms in iTau-HEK cells, we found that iCLU interacted exclusively with BIN1 isoforms 1–3, suggesting that association between iCLU and specific BIN1 isoforms depended on the presence of a coiled-coil motif (Fig. 4a). Despite the selective interaction between iCLU and BIN isoforms 1–3, all 10 isoforms of BIN1 co-immunoprecipitated with Tau. These results suggested that BIN1 interaction with Tau is not mediated by the coil-coiled motif or the CLAP domain of BIN1, and it is independent of
interaction with iCLU. Next we further examined iCLU, BIN1 isoform 1 (BIN1.1) and Tau interactions in iTau-HEK cells. Upon co-transfection of iCLU and BIN1.1 in iTau-HEK cells, association of all three proteins was observed when lysates were immunoprecipitated with anti-Tau antibody, either upon overexpression of Tau (DOX) or with endogenous levels of Tau (PBS) (Fig. 4b). Independent of dox treatment, pull-down of cell lysates with anti-BIN1 antibody led to co-immunoprecipitation of iCLU, but co-immunoprecipitated Tau was detected only in dox-treated cells (Fig. 4c). When anti-CLU antibody was used for the immunoprecipitation step, BIN1.1 was pulled-down independently of dox treatment (Fig. 4d). Overall, these results suggested that the interaction between iCLU and BIN1.1 appears to occur independently of their association with Tau. In summary, our cellular findings suggested that two important genetic susceptibility factors for AD, BIN1 and CLU, are interacting proteins that also bind to the microtubule-associated protein Tau.

CLU interacts with BIN1.1 and with modified Tau species in AD brains

The interaction between CLU, BIN1 and Tau was further examined in brain tissue from control and AD patients. Direct Western blotting analysis showed that, in addition to the precursor and sCLU, iCLU isoforms were also detected in the human brain (Fig. 5a). BIN1 isoforms 1, 2, 3, 7, 9 and 10 were detected in whole brain homogenates (Fig. 5a). Further analysis of human neuron and astrocyte primary cell cultures revealed that iCLU forms are detected in both brain cell types (Fig. 5b). In addition, the longer brain-specific BIN1 isoforms 1–5 were predominantly detected in neurons, while the shorter ubiquitous isoforms 9 and 10 were mainly found in astrocytes (Fig. 5c). Subcellular fractionation of human brain tissue indicated that intracellular forms of CLU were preferentially associated with the cytoskeleton/insoluble fraction from brain extracts of both AD and control subjects (Fig. 5d). While most of BIN1.1 was detected in the cytosolic and membrane fractions, a significant amount of BIN1.1 was found in the cytoskeleton/insoluble fraction from control and diseased human brain (Fig. 5d). In AD brains, but not in controls, Tau and p-Tau species were also localized to the cytoskeleton/insoluble fraction (Fig. 5d).

Since iCLU, BIN1, and Tau were all co-localized to the cytoskeleton/insoluble fraction in the AD brain, we sought to confirm our cellular findings using the co-immunoprecipitation approach with human brain lysates. While input fractions from both disease (n = 3) and control brains (n = 3) were comparable for all proteins analyzed, immunoprecipitation of brain lysates with anti-CLU antibody showed increased pull-down of BIN1.1 and Tau in AD compared to controls (Fig. 6a). Similarly, more CLU, Tau and p-Tau were immunoprecipitated from AD than from control brain lysates with anti-BIN1 antibody (Fig. 6b). Finally, immunoprecipitation of lysates with anti-Tau antibody led to greater pull-down of BIN1.1 and CLU from AD than from control tissue (Fig. 6c). Together, these findings support a relevant interaction between CLU, BIN1.1 and Tau in the AD brain.

Rare coding CLU variants associated with AD alter sCLU/iCLU ratio

Finally, we evaluated the interaction between BIN1.1 and eight rare CLU variants recently associated with AD [8]. The coding point mutations were introduced in the iCLU WT flag-tagged construct by site-directed mutagenesis (Fig. 7a). While CLU mutants 1–7 appear to interact equally with BIN1.1, a frame-shift CLU
mutant lacking the C-terminus coiled-coil region failed to co-immunoprecipitate with BIN1.1 (Fig. 7b) further corroborating that iCLU and BIN1 association occurs via a coiled-coil interaction. Moreover, overexpression of full-length CLU mutants 1–7 in HEK 293T cells showed that certain coding mutations (MT1 and MT6) were associated with increased generation of iCLU relative to sCLU (Fig. 7c), suggesting a potential mechanism for pathogenicity of these variants.

Discussion

After APOE, the CLU and BIN1 genes have been identified as the most important susceptibility loci in late-onset AD [1–7,33–37]. It has been hypothesized that CLU is involved in amyloid clearance, playing a protective role in AD. However, contrary to this premise, plasma levels of CLU correlate positively with AD severity and progression [50], and increased CLU mRNA expression is associated with a more deteriorated disease status [51]. It has been recently reported that elevated CSF clusterin contributes to entorhinal atrophy in patients with mild cognitive impairment and increased Aβ42 deposition [52], suggesting that clusterin might be involved in AD pathogenesis. In the present study we found a link between the CLU rs11136000 SNP and CSF Tau levels in AD patients and provided evidence that intracellular forms of clusterin might play an important role in AD pathology. While the secreted form of CLU (sCLU) is induced during stress and inflammation and believed to be protective, intracellular forms of CLU previously linked to cytotoxicity are also upregulated under these conditions [12,14]. Mechanisms induced by cellular stress, such as abnormal translocation of CLU from the ER/Golgi to the cytosol [12] and impaired degradation by the proteosome [14], as well as increased Aβ levels [17] and scrapie infection [53], all appear to drive accumulation of iCLU. Therefore CLU mutations that alter the ratio between iCLU and sCLU produced during stress could potentially modify risk for AD.

In support of this hypothesis, we showed that certain AD-risk mutations in CLU significantly increased the ratio iCLU/sCLU following expression of the full-length CLU construct in vitro.

We reported here that overexpression of human Tau in the mouse brain was associated with a marked increase in iCLU levels before any pathological forms of Tau were detected, suggesting that iCLU and Tau are predicted to interact in vivo. However, because Tg4510 mice overexpress the frontotemporal dementia mutant P301L Tau, which has reduced ability to promote microtubule assembly and is more prone to aggregation than WT Tau [54] found in normal and AD patients, we performed reciprocal co-immunoprecipitation experiments comparing the interaction between iCLU and WT or P301L Tau in HEK 293T cells. Our results indicated that both Tau forms co-immunoprecipitated with iCLU when co-expressed in a cellular system and validated our conclusion that increased iCLU expression in Tg4510 mouse brain is indeed suggestive of a physiologically relevant interaction between iCLU and Tau.

Figure 3. iCLU localizes to the cytoskeleton fraction and is a Tau-interacting protein. (a) Subcellular fractionation of HEK 293T after transfection with full-length CLU cDNA indicated that most of iCLU was found in the cytoskeleton/insoluble fraction. (b) Transfection of sCLU or iCLU cDNA in Tau-inducible HEK293 cells (iTau-HEK) followed by pull-down with anti-Tau antibody (HT7) showed co-immunoprecipitation of iCLU. (c) Transient co-transfection of Flag-tagged iCLU construct with WT or P301L mutant full-length Tau in HEK 293T cells followed by pull-down with anti-Tau antibody (HT7) or anti-Flag antibody showed reciprocal co-immunoprecipitation of iCLU and Tau, respectively. Conversely, transient co-transfection of Flag-tagged iCLU with WT or P301L 4-repeat microtubule binding domain (MBD) of Tau alone did not result in co-immunoprecipitation of Tau by anti-Flag antibody.

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Our cellular data supporting CLU as a novel Tau-interacting protein is similar to recent findings reported for BIN1 [39]. While iCLU interacted with both WT and P301L mutant full-length Tau, it did not co-immunoprecipitate the 4-repeat MBD of Tau alone, suggesting that the interaction between iCLU and Tau occurs outside the microtubule binding region. In addition, we showed that CLU and BIN1 co-immunoprecipitated primarily with Tau species found in neurofibrillary tangles from AD brains. Because initial findings from Chapuis et al. [39] suggest that BIN1 expression is detrimental, and increased expression of both CLU and brain-specific isoforms of BIN1 has been associated with AD status [51], we speculate that this newly discovered interaction between CLU and BIN1 could be related to AD risk via modulation of Tau function. CLU was previously shown to also interact via its coil-coiled motif with SCLIP, a member of the stathmin family proteins that modulate microtubule dynamics through association with tubulin [49]. Therefore, BIN1 expression levels could potentially modulate the interaction between iCLU and proteins regulating microtubule dynamics, including Tau.

A new interaction between CLU and coiled-coil-containing isoforms of neuronal BIN1 demonstrated here uncovers other putative mechanisms by which the interaction between iCLU and Tau could affect AD risk. It has been recently reported that the coiled-coil motif of neuronal BIN1 isoforms is also required for binding to dynamin 2, and that this interaction is essential for endocytosis [55]. Therefore, we hypothesize that increased intracellular CLU could also impair endocytosis by interfering with the interaction between BIN1 and dynamin 2. Endocytosis appears to be crucial in the etiology of AD as SNPs in genes encoding endocytosis-related proteins have been linked to the disease, including the clathrin adaptor PICALM [1–6] and CD2AP [36]. Interestingly, both PICALM and CD2AP have also been recently linked to Tau pathology [56,57]. PICALM was found to co-localize and co-immunoprecipitate with hyperphosphorylated and misfolded Tau from brains of AD patients [53], while CD2AP was found to modulate Tau toxicity in Drosophila [57]. Knockdown of cindr, the fly ortholog of the human CD2AP, enhanced Tau-induced retinal toxicity [57], similarly to results observed for the fly ortholog of BIN1 [39].

In summary, our novel findings provide groundwork for future studies that could shed light on the mechanisms by which GWAS-identified genes CLU and BIN1 are linked to Tau pathology in AD, and help uncover new areas for therapeutic intervention.

**Materials and Methods**

**Reagents and Antibodies**

Reagents were purchased from Sigma-Aldrich unless specified otherwise. Antibodies used in this study were as follows: anti-APP 6E10 (SIG-39320, Covance), anti-Aβ 12F4 (SIG-39142, Covance), mouse anti-Tau Tau12 (SIG-39416, Covance), mouse anti-Tau HT7 (MN1000, Thermo Scientific Pierce), mouse anti-phosphorylated Tau AT8 (MN1020, Thermo Scientific Pierce), rabbit anti-Tau H150 (SC-5587, Santa Cruz Biotechnology), rabbit anti-Tau H17 (MN1000, Thermo Scientific Pierce), mouse anti-clusterin M18 (SC-6420, Santa Cruz Biotechnology), goat anti-clusterin C18 (SC-6419, Santa Cruz Biotechnology), mouse anti-BIN1 99D (05-449,

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**Figure 4.** iCLU interacts with coiled-coil-containing BIN1 isoforms. (a) Co-expression of each of the ten BIN1 human isoforms with iCLU in iTau-HEK cells revealed that iCLU interacted only with brain-specific BIN1 isoforms 1–3 containing a putative coiled-coil motif within the BAR domain (black arrows); however, all isoforms of BIN1 showed interaction with Tau (white arrows). (b) Western blotting of lysates immunoprecipitated with anti-Tau HT7 antibody indicated that both iCLU and BIN1.1 were co-immunoprecipitated from both PBS and dox-treated cells. (c) Western blotting from BIN1 99 antibody pull-down showed co-precipitation of iCLU in both PBS and dox conditions, but Tau bands were only detected in dox-treated cells. (d) Western blotting of anti-CLU C18 antibody immunoprecipitates showed similar bands for BIN1.1 in PBS and dox-treated cells.

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Millipore), rabbit anti-Gapdh (G9545), rabbit anti-actin (A2066),
and mouse anti-Flag (F1804), mouse anti-myc 9E10 (SC-40, Santa
Cruz Biotechnology), mouse and goat IgG control (SC-2015 and
SC-2028, Santa Cruz Biotechnology), donkey anti-mouse IgG (H
+ L) IRDye 800 (926-32212, LI-COR Biosciences), donkey anti-
rabbit IgG (H + L) IRDye 680 (926-68073, LI-COR Biosciences),
donkey anti-rabbit IgG (H + L) IRDye 800 (926-32213, LI-COR
Biosciences), and donkey anti-goat IgG (H + L) IRDye 680 (926-
68074, LI-COR Biosciences).

Human Samples and Genotyping
All human samples were obtained from commercial sources and
analyzed anonymously. Human blood and cerebrospinal fluid
(CSF) from clinically diagnosed Alzheimer’s disease patients and
aged controls were purchased from PrecisionMed, Inc (www.
precisionmed.com). CSF samples were aliquoted and stored at
−80°C until analysis. Genomic DNA was extracted from blood cells
using the QIAamp DNA blood midi prep (Qiagen). DNA samples
were stored at −20°C until used. APOE e2/e3/e4 and CLU
rs11136000 polymorphisms were determined using polymerase
chain reaction and restriction fragment length polymorphism
(PCR-RFLP) method. All primers used for PCR were purchased
from Applied Biosystems. PCR reagents included 2.5 μl of
AmpliTaq Gold 360 Master Mix (Applied Biosystems). For
APOE genotyping, forward and reverse primer sequences were:
5′-ACAGAATTCTGGCCCACCCTGGTACAC 3′ and 5′-
TAAGGCTGGCACAGGCTTGCCAAAGGA 3′. PCR products
were digested with HhaI (New England Biolab) at 37°C for
1 hr, and the digested products were run in 20% TBE
polyacrylamide gels. For genotyping the CLU rs11136000
polymorphism, forward and reverse primer sequences were:
5′-CTTTGAATGATGTACCATCTACCC 3′ and 5′-
AGGCTGCCAGACTCCCTGAAT 3′. PCR products were digested with
ApoI (New England Biolab) at 37°C for 1 hr, and the digested
products were run in 8% TBE polyacrylamide gels. Human brain
tissue was obtained from National Disease Research Interchange
(NDRI) and stored at −80°C until analysis.

Quantification of CSF Aβ42, Tau and Clusterin
CSF Aβ42 was measured by a sandwich ELISA system using
6E10 as a capture antibody and alkaline phosphatase (AP-
conjugated 12F4 as a detection antibody. A 96-well black plate
(Corning) was coated with the 6E10 monoclonal antibody at the
concentration of 2 µg/ml in 0.05 M carbonate-bicarbonate buffer,
ph 9.6, incubated overnight at 4°C with shaking. washed with
PBS containing 0.05% Tween 20 and blocked with PBS
containing 3% bovine serum albumin (BSA) for at least 24 hr at
4°C with mixing. CSF was diluted at 1:4 with PBS containing 3% BSA. Diluted samples and a standard series of synthetic Aβ42 peptide (American Peptide Company) were incubated in triplicates with AP-conjugated detection antibody at 4°C overnight with shaking. After incubation, plates were washed and incubated with CDP-Star chemiluminescent substrate (Applied Biosystems) at RT for 20 min. The chemiluminescence was measured with an EnVision plate reader (Perkin Elmer). CSF total Tau was measured with INNOTEST hTAU Ag (Innogenetics) according to manufacturers’ instructions. CSF clusterin (1:200 dilution) was measured using a human clusterin ELISA kit (DCLU00, R&D Systems) according to manufacturers’ instructions.

Animals
Tg4510 human Tau and Tg2576 human APP transgenic mouse lines were maintained at Taconic. Brains harvested from animals immediately after euthanasia with carbon dioxide were flash-frozen and stored at −80°C for subsequent analysis. All animals were handled according to the Public Health Service Policy on Humane Care and Use of Laboratory Animals guidelines and the study protocol was approved by the Institutional Animal Care and Use Committee of Merck Research Laboratories ( Permit Number: 12089204580220).

Plasmid cDNA
The pCMV6-XL5 empty vector was obtained from Origene. Full-length and intracellular human clusterin (FL CLU and iCLU, respectively) constructs were generated from human clusterin variant 1 cDNA clone (SC118977) purchased from Origene. Briefly, the first 52 and 85 amino acids of human clusterin isoform 1 (MQVC…RIGG and MQVC…ELQE, respectively) were deleted to generate FL CLU and iCLU, respectively, and the FLAG tag sequence (DYKDDDDK) was added at the C-terminus. Human BIN1 cDNA clones in a pCMV6-entry vector with myc-tag sequence (TRTRPLEQEKLISEEDLAANDILDYKDDDDKV) at the C-terminus were obtained from Origene. Tau constructs (4R2N WT, 4R2N P301L, K18 WT and K18 P301L) were generated in house by subcloning into pcDNA4/TO vector from Life Technologies.
Cell Culture and Transfection
Parental HEK 293T cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies). HEK 293T cells stably expressing inducible human Tau 4R2N (iTau-HEK) were developed at Origene. iTau-HEK cells were maintained in DMEM containing 10% Tet system approved FBS (Clontech), 16 non-essential amino acids (Life Technologies), 200 mg/mL hygromycin B (Life Technologies) and 400 mg/mL geneticin (Life Technologies) at 37°C with 5% CO2. Tau expression was induced by incubating iTau-HEK cells in growth media containing 1 mg/mL doxycycline (Clontech) for 48–72 hr. Transient transfections of CLU and Tau constructs were performed using the Lipofectamine LTX reagent (Life Technologies) according to the manufacturer’s instructions. Cells were harvested 48–72 hr after transfection for subsequent analyses.

Subcellular fractionation
Subcellular fractionation of cells and brain tissue was performed using the Qproteome Cell Compartment Kit (Qiagen) according to the manufacturer’s instructions. Briefly, cell pellets (∼5 x 10^6 cells) or brain tissue (20–30 mg) were solubilized in lysis buffer and centrifuged at 1,000 g for 10 min at 4°C. Cytosolic proteins were obtained from the supernatant. Subsequent pellet was resuspended in benzamidine nuclease and extraction buffer CE3 and centrifuged at 6,800 g for 10 min at 4°C. Nuclear proteins were obtained from the supernatant. The final pellet was resuspended in extraction buffer CE4 to obtain cytoskeletal/insoluble proteins.

Western blotting and Immunoprecipitation
Tissue samples were homogenized in NP-40 lysis buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 0.5% NP40) or in Triton lysis buffer containing 1% Triton X-100 in phosphate buffered saline (PBS) supplemented with Complete protease inhibitor cocktail (Roche Applied Sciences) and PhosSTOP phosphatase inhibitor cocktail (Roche Applied Sciences) using a TissueLyzer. Cells were washed briefly with ice-cold PBS and lysed in NP-40 or Triton lysis buffer supplemented with Complete protease inhibitor and PhosSTOP phosphatase inhibitor cocktails. All lysates were solubilized at 4°C for 1–3 hr and cleared by centrifugation at 10,000 g for 10 min. Protein concentration was determined by the BCA protein assay kit (Pierce). For Western blotting analysis, lysates were heated at 70°C for 10 min in NuPAGE LDS sample buffer and NuPAGE sample reducing agent (Life Technologies), and resolved on NuPAGE Novex Bis-Tris gel (Life Technologies). Membranes were immunoblotted with appropriate antibodies and analyzed with the Odyssey infrared imaging system (LI-COR). For immunoprecipitation, protein G-coated dynabeads (Life Technologies) were coated with appropriate antibodies at a concentration of 5 µg of antibody per 50 µl of dynabeads. Tissue or cell lysates from the supernatant. Subsequent pellet was resuspended in benzamidine nuclease and extraction buffer CE3 and centrifuged at 6,800 g for 10 min at 4°C. Nuclear proteins were obtained from the supernatant. The final pellet was resuspended in extraction buffer CE4 to obtain cytoskeletal/insoluble proteins.
were incubated with antibody-coated dynabeads for 30 min at RT. After immunoprecipitation, samples were suspended in 1x NuPAGE LDS sample buffer and NuPAGE sample reducing agent and analyzed by Western blotting.

Primary neuronal and astrocyte cultures

Human neurons (Cat# 1520) and astrocytes (Cat# 1801) were obtained from ScienCell Research. Briefly, neurons were plated in 6-well poly-L-lysine-coated culture plates (Thermo 125035) according to manufacturers' protocol. After 14 days in culture, neurons were washed with CBM-PRP medium and lysed in RIPA buffer (Thermo 89900) supplemented with Complete protease inhibitor and PhosSTOP phosphatase inhibitor cocktails (Roche Applied Sciences). For human astrocytes, cells were cultured following manufacturer instructions and 0.5×10⁶ cells were subcultured in 6-well poly-L-lysine-coated culture plates for 24 hrs. Astrocytes were then washed and lysed in the same manner as human neurons. Lysates were solubilized at 4°C for 1 hr and cleared by centrifugation at 16,000 g for 5 min. Protein concentration was determined by the BCA protein assay kit (Pierce).

Statistical analysis

Receiver operating characteristic curve (ROC) analysis was performed using JMP 11 (SAS Institute). Group differences for each analyte were assessed by the Student’s t test for two-group comparisons and the One-way ANOVA test for multiple comparisons using GraphPad Prism 5.0 (GraphPad Software Inc.). For comparison of categorical parameters, the Pearson’s chi-square test was used. A P value of less than or equal to 0.03 was considered statistically significant.

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Author Contributions

Conceived and designed the experiments: CZ YZ IH. Performed the experiments: YZ IH JW KT. Analyzed the data: IH YZ CZ. Contributed reagents/materials/analysis tools: JR. Wrote the paper: CZ YZ IH.

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