Cleavage of tau by asparagine endopeptidase mediates the neurofibrillary pathology in Alzheimer’s disease

Zhentao Zhang1,2, Mingke Song3, Xia Liu1, Seong Su Kang1, Il-Sun Kwon1, Duc M Duong4,5, Nicholas T Seyfried4,5, William T Hu6, Zhixue Liu7, Jian-Zhi Wang8, Liming Cheng9, Yi E Sun9, Shan Ping Yu3, Allan I Levey5,6 & Keqiang Ye1

Neurofibrillary tangles (NFTs), composed of truncated and hyperphosphorylated tau, are a common feature of numerous aging-related neurodegenerative diseases, including Alzheimer’s disease (AD). However, the molecular mechanisms mediating tau truncation and aggregation during aging remain elusive. Here we show that asparagine endopeptidase (AEP), a lysosomal cysteine protease, is activated during aging and proteolytically degrades tau, abolishes its microtubule assembly function, induces tau aggregation and triggers neurodegeneration. AEP is upregulated and active during aging and is activated in human AD brain and tau P301S–transgenic mice with synaptic pathology and behavioral impairments, leading to tau truncation in NFTs. Tau P301S–transgenic mice with deletion of the gene encoding AEP show substantially reduced tau hyperphosphorylation, less synapse loss and rescue of impaired hippocampal synaptic function and cognitive deficits. Mice infected with adeno-associated virus encoding an uncleavable tau mutant showed attenuated pathological and behavioral defects compared to mice injected with adeno-associated virus encoding tau P301S. Together, these observations indicate that AEP acts as a crucial mediator of tau-related clinical and neuropathological changes. Inhibition of AEP may be therapeutically useful for treating tau-mediated neurodegenerative diseases.

AD is a progressive neurodegenerative disease characterized by two neuropathological hallmarks: extracellular senile plaque deposits, composed of amyloid beta (Aβ), and intracellular NFTs, made of truncated and hyperphosphorylated tau. Tau-mediated neurodegeneration may result from the combination of toxic gains of function acquired by the aggregates and the detrimental effects that arise from the loss of the normal functions1. Tau is mainly expressed in neurons and abundant in the neuronal axons, regulating microtubule (MT) polymerization and stabilizing MTs. Through alternative splicing, the MAPT gene yields six major isoforms of tau2. Whereas normal phosphorylation of tau controls the dynamics of MT, establishing neuronal polarity, axonal outgrowth and axonal transport3–6, pathological hyperphosphorylation in disease severely interferes with tau’s ability to regulate MT dynamics7–9. Hyperphosphorylated tau displays an increased propensity to form paired helical filaments (PHFs) in vitro and sequesters full-length tau and other MT-associated proteins10–12, indicating that hyperphosphorylation is a potent inducer of tau pathology. In AD, tau undergoes a number of other post-translational modifications in addition to phosphorylation that contribute to the tau aggregation and disease pathology.

Tau is a substrate for various proteases. It can be cleaved by several caspasas at D421. In AD brain, tau truncated at Asp421 is a component of NFTs, and Aβ induces this cleavage in cultured neurons13–15. In AD brain, calpain 1 and calpain 2 are abnormally activated16. Aβ treatment leads to activation of calpains and production of a 17-kDa fragment in neurons (tau45−230). Overexpression of tau45−230 induces neuronal apoptosis17. In addition to caspasas and calpains, thrombin and cathepsins have also been implicated in processing tau18–20. However, many tau fragments found in AD are not well characterized, and the proteases responsible for their generation have not all been identified. For instance, a tau fragment of 25–35 kDa in the cerebrospinal fluid (CSF) has been used as an early marker of AD21,22, but the proteases responsible for this cleavage event are unknown.

Mammalian AEP, also known as legumain (LGMN), is a lysosomal cysteine protease that cleaves protein substrates on the C-terminal side of asparagine23,24. AEP activation is autocatalytic and requires sequential removal of C- and N-terminal propeptides at different pH thresholds25. Recently, we showed that neuronal AEP is involved in neuronal apoptosis through its degradation of the DNase inhibitor SET during excitoneurotoxicity26 and that AEP cleaves TDP-43.

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1Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia, USA. 2Department of Neurology, Renmin Hospital of Wuhan University, Wuhan, China. 3Department of Anesthesiology, Emory University School of Medicine, Atlanta, Georgia, USA. 4Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia, USA. 5Center for Neurodegenerative Diseases, Emory University School of Medicine, Atlanta, Georgia, USA. 6Department of Neurology, Emory University School of Medicine, Atlanta, Georgia, USA. 7Department of Pathophysiology, Key Laboratory of Ministry of Education of Neurological Diseases, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. 8Translational Center for Stem Cell Research, Tongji Hospital, Department of Regenerative Medicine, Tongji University School of Medicine, Shanghai, China. Correspondence should be addressed to L.C. (limingcheng@tongji.edu.cn) or K.Y. (kye@emory.edu).
in post-mortem brain from humans with frontotemporal lobar degeneration. In this report, we show that AEP cleaves tau at both N255 and N368 residues, induces tau aggregation and attenuates its MT-stabilizing activity. Furthermore, AEP is highly activated in tau P301S-transgenic mice and human AD brains. Consistent with this, an AEP-cleaved tau fragment at N368 is detected in human AD brains. Knockout of AEP in tau P301S mice leads to a reduction of tau hyperphosphorylation, protecting against memory loss. Blockade of tau cleavage by AEP rescues tau P301S–triggered pathologic and behavioral defects. Hence, our results suggest that tau is a physiological substrate of AEP and that AEP is a mechanism-based therapeutic target for treating tauopathies including AD.

RESULTS

AEP directly cleaves tau

To explore whether AEP proteolytically processes tau, we conducted an in vitro cleavage assay with kidney lysates prepared from wild-type (Lgmn+/+) and Lgmn–/– mice under pH 7.4 or 6.0. At pH 6.0, AEP was active, cleaving tau into two fragments, whereas at pH 7.4 AEP was inactive (Fig. 1a). AEP enzymatic activity in kidney lysates was confirmed by an activity assay (Fig. 1a). When cotransfected with GST-tau, wild-type AEP strongly triggered tau fragmentation, whereas AEP proteins with mutations that abolish the cysteine protease activity of AEP were inactive, tau remained intact regardless of AEP genotypes (Fig. 1d). We identified two partial-tryptic peptides ending with N255 and N368 from LC-MS/MS (Fig. 2b). The amino acid number is based on the longest tau isoform (isoform 2, amino acids 1–441). To avoid unnecessary confusion, we adopted isoform 2’s numbering system, though for essential confusion, we adopted isoform 4’s numbering system. A biochemical assay with purified active AEP and recombinant GST-tau revealed that purified AEP indeed potently and directly cleaved tau (Fig. 1e). Moreover, tau was degraded in both Lgmn+/+ and Lgmn–/– mice at pH 6.0, but this effect was substantially attenuated in Lgmn–/– mice. By contrast, at pH 7.4, where AEP is inactive, tau remained intact regardless of AEP genotypes (Fig. 1f). AEP enzymatic activities were also validated by fluorescent substrate cleavage assay (Fig. 1g). Together, these results strongly support that tau is a direct substrate of AEP.

AEP cleaves endogenous tau at N255 and N368

To identify the AEP cleavage sites on tau, we purified the fragmented GST-tau proteins with molecular weights of approximately 50 and 60 kDa and performed mass spectrometry analysis (Fig. 2a). We identified two partial-tryptic peptides ending with N255 and N368 from LC-MS/MS (Fig. 2b). The amino acid number is based on the longest tau isoform (isoform 2, amino acids 1–441). To avoid unnecessary confusion, we adopted isoform 2’s numbering system, though for essential confusion, we adopted isoform 4’s numbering system. A biochemical assay with purified active AEP and recombinant GST-tau revealed that purified AEP indeed potently and directly cleaved tau (Fig. 1e). Moreover, tau was degraded in both Lgmn+/+ and Lgmn–/– mice at pH 6.0, but this effect was substantially attenuated in Lgmn–/– mice. By contrast, at pH 7.4, where AEP is inactive, tau remained intact regardless of AEP genotypes (Fig. 1f). AEP enzymatic activities were also validated by fluorescent substrate cleavage assay (Fig. 1g). Together, these results strongly support that tau is a direct substrate of AEP.

Figure 1 AEP cleaves tau in vitro. (a) Tau cleavage assay. Left, western blot showing the cleavage of GST-tau after incubation with kidney lysates from Lgmn+/+ or Lgmn–/– mice at pH 7.4 or pH 6.0. Right, AEP activity assay. Data represent mean ± s.e.m. of three independent experiments. MW, molecular weight. AFU, arbitrary fluorescence units. mGST, mammalian glutathione S-transferase. (b) Western blot showing the cleavage of tau by wild-type (WT) and mutant AEP. (c) Top, western blot showing the effect of AENK and AEQK on tau cleavage. Bottom, AEP activity assay. Data represent mean ± s.e.m. of three independent experiments. (d) Antibody titration assay. Mouse IgG was used as negative control. (e) Western blot showing the cleavage of purified GST-tau recombinant protein by purified active recombinant AEP. (f) Western blot showing the cleavage of endogenous tau by AEP. Western blot data in a–f are representative of three independent experiments. (g) Validation of AEP enzymatic activities by fluorescent substrate cleavage assay. Data represent mean ± s.e.m. of three independent experiments.
the presence of active AEP (Fig. 2c), suggesting that N255 and N368 are the two major AEP cleavage sites on tau.

Moreover, we identified peptide N368 in human AD brain samples by mass spectrometry (Fig. 2d), suggesting that tau cleavage at N368 by AEP occurs in human AD brain. Finally, we identified both N255 and N368 peptides in whole mouse brain extracts, and comparative label-free proteomic analysis revealed >20-fold enrichment for both N368 and N255 in Lgmn<sup>−/−</sup> versus Lgmn<sup>+/+</sup> brain extracts (Fig. 2e). Hence, tau is a physiological substrate of AEP, which cleaves tau at both N255 and N368 sites.

**AEP cleaves tau independent of caspases or calpains**

Tau can be proteolytically processed by numerous proteinases including caspases, calpains, cathepsins, thrombin and puromycin-sensitive aminopeptidase (PSA)28. We assessed the effect of their specific inhibitors on the processing of tau by AEP and found that tau cleavage was selectively reduced by the specific AEP inhibitor AENK but not by any of the other protease inhibitors (Supplementary Fig. 1a). Remarkably, tau with point mutations of the cleavage sites (N255A/N368A) remained intact (Supplementary Fig. 1b). These results indicate that cleavage by other proteases is not necessary for AEP processing of tau.

On the other hand, calpain cleavage analysis revealed that tau wild-type, N255A/N368A mutant and tau fragment (1–255) were cleaved in a time-dependent manner with consistent rates (Supplementary Fig. 1c), indicating that AEP cleavage of tau does not affect proteolytic processing by calpains. We made a similar observation with both...
AEP is upregulated and cleaves tau during aging and in AD. (a) Western blot analysis of tau and AEP in mouse brain during aging process. (b) AEP activity assay. Data represent mean ± s.e.m. of 6 mice per age; *P < 0.05 compared with 1- and 2-month-old mouse brains. **P < 0.01 compared with 1-, 2-, and 4-month-old mouse brains, one-way analysis of variance (ANOVA). (c) Western blot detection of tau fragments in Lgmn−/− and Lgmn+/+ mouse brain. (d) Immunohistochemistry (IHC) of tau N368 fragments in brain sections from subjects with AD and control subjects (*P < 0.01, Student’s t-test). Data represent mean ± s.e.m. of 18–20 sections from 3 samples each group. Scale bar, 50 µm. (e) Western blot detection of tau fragments in human brain samples from six subjects with AD and three age-matched controls. (f) Immunostaining showing colocalization of tau N368 fragment with PHFs. Brain sections from subjects with AD were immunostained with anti–tau N368 antibody and then stained with thioflavin S, which labels both the senile plaque (arrowheads) and PHFs (arrows). Scale bar, 50 µm. Images are representative of 9 sections from three subjects with AD. (g) AEP activity assay in brain samples from subjects with AD and age-matched controls (mean ± s.e.m.; representative data of four independent experiments with 3 control and 6 AD samples, *P < 0.05 compared with control group, one-way ANOVA). (h) AEP activity assay in 6-month-old tau P301S–transgenic mice and nontransgenic controls (mean ± s.e.m.; n = 6 mice per group; *P < 0.01, Student’s t-test). (i) pH in the brain cortex and hippocampus of control and tau P301S–transgenic mice (mean ± s.e.m.; n = 6 mice per group, *P < 0.01, Student’s t-test). Western blot data in a, c, and e are representative of three independent experiments.

AEP is activated during the aging process and in human AD brains

As age is the major risk factor for AD and the pH in the brain gradually decreases during aging, we sought to investigate tau degradation by AEP in the mouse brain at different ages. Tau was fragmented progressively with age, with degradation products detectable as early as 8 months of age (Fig. 3a). To further investigate tau fragmentation by AEP, we developed an anti–tau N368 antibody, which specifically recognized the cleaved tau 1–368 band but not full-length tau or tau 1–255 band (Supplementary Fig. 2a,b). The bands immunoprecipitated by anti–tau N368 antibody were recognized by two different tau-specific antibodies (tau1 and tau5), suggesting that AEP-cleaved tau but not other proteins was selectively immunoprecipitated (Supplementary Fig. 1c). We detected tau immunoreactive fragments in 8- and 13-month-old mouse brains with anti–tau N368 (Fig. 3b). In agreement with this finding, we found that AEP activity also increased with aging, correlating with the tau cleavage pattern (Fig. 3b). The tau fragmentation was completely abolished in Lgmn−/− brain, supporting the idea that tau degradation during aging is mediated by AEP (Fig. 3c). The anti–tau N368 antibody also robustly labeled tau N368 immunoreactivity in human AD brain sections, whereas the signals were markedly attenuated in age-matched control brains (Fig. 3d). Preincubation with the antigen peptide (tau 360–368) ablated immunoreactivity (Supplementary Fig. 2d), supporting the specificity of the anti–tau N368 antibody. Furthermore, we found that tau N368 immunoreactive fragments were abundant in human AD brains but barely detectable in controls (Fig. 3e). Moreover, immunofluorescence staining revealed that tau N368 colocalized with thioflavin S–positive NFTs in human AD brains (Fig. 3f), and tau N368 also colocalized with phosphorylated tau (Supplementary Fig. 2e), indicating that AEP-induced fragments of tau are constituents of wild-type and tau N255A/N368A mutant in a caspase-3 cleavage assay (Supplementary Fig. 1d). Thus, AEP and other proteases can independently fragment tau. To test whether tau hyperphosphorylation influences tau cleavage by AEP, we pretreated GST-tau–transfected HEK293 cells with the protein phosphatase PP2 inhibitor okadaic acid and monitored tau fragmentation. GST-tau was cleaved in a time-dependent manner regardless of okadaic acid treatment (Supplementary Fig. 1e), indicating that hyperphosphorylation of tau does not interfere with AEP cleavage.
AEP (255) TAU FL Roscovitine SB216763 H89 Control

Figure 4 Tau cleavage by AEP disrupts its MT assembly activity and is toxic to neurons. (a) Schematic diagram of tau isoform 4 domains and its cleavage by AEP. I, Inserts; P, proline rich; R, repeats; R′, pseudo-repeat; C, C-terminal tail; FL, full length. (b) Representative western blot of purified His-tagged tau fragments (two independent experiments). (c) MT assembly assay. Data represent mean of three independent experiments. (d) Axon elongation in primary neurons transfected with control plasmid, HA-tau or HA-tau fragments (mean ± s.e.m. of five independent experiments; *P < 0.05, one-way ANOVA). (e,f) TUNEL assay showing the neurotoxicity of AEP-derived tau fragments. Data represent the mean ± s.e.m. of three independent experiments; *P < 0.01, one-way ANOVA. Scale bar, 10 μm. (g) Effect of kinase inhibitors on the neurotoxic effect of tau 1–368. Primary neurons infected with AAV–tau 1–368 were treated with 10 μM roscovitine (CDK5 inhibitor), SB216763 (GSK3 inhibitor) or H89 (PKA inhibitor) for 12 h. Cell apoptosis was detected by TUNEL staining. Data represent mean ± s.e.m. of four independent experiments. Scale bar, 20 μm.

NFTs in vivo. AEP enzymatic activities in human AD brains were also higher than in controls (Fig. 3g), fitting with the marked tau N368 fragmentation in human AD samples. Further, AEP activity was elevated in tau P301S–transgenic mice compared to in age-matched control nontransgenic mice (Fig. 3h). As expected, the pH in the transgenic mouse brain cortex and hippocampus was lower compared to in nontransgenic controls (Fig. 3i). Hence, AEP is activated in aging mouse brain, and it is also activated in both human AD brains and tau P301S–transgenic mice.

Noticeably, Aβ oligomers elicited both AEP and tau fragmentation in a dose-dependent manner in primary rat neuronal cultures (Supplementary Fig. 3a,b). AEP was dose-dependently activated by Aβ, whereas cathepsin B, an AEP-related cysteine protease, was not activated. As expected, caspase-3 activity increased with Aβ in a concentration-dependent manner (Supplementary Fig. 3c–e). Moreover, overexpression of AEP in primary neurons elicited demonstrable tau N368 degradation (Supplementary Fig. 3f). Thus, Aβ may provoke AEP activation, resulting in tau degradation at N368.

AEP cleavage of tau generates neurotoxic fragments

to assess whether AEP cleavage may affect the functions of tau in promoting MT polymerization, we conducted an in vitro MT polymerization assay with purified tubulin in the presence of His-tagged tau fragments. As expected, full-length tau strongly augmented MT polymerization; in contrast, the stimulatory effects of truncated tau fragments 1–368 and 256–441 were greatly reduced. Although tau fragment 256–368 moderately increased MT polymerization in vitro, tau fragments 1–255 and 369–441 failed to induce polymerization (Fig. 4a–c). Accordingly, overexpression of full-length tau in primary neurons notably increased axon length; in contrast, axon elongation was not enhanced in neurons expressing any one of the tau fragments (Fig. 4d).

In neurons transfected with a mixture of the tau fragments, apoptosis was increased more than 60% compared to neurons transfected with full-length tau (Fig. 4e). TUNEL staining showed that the tau fragments 1–368 and 256–368 triggered substantial apoptosis compared to wild-type or other tau fragments (Fig. 4f). These results were confirmed in neurons infected with adeno-associated viruses (AAVs) encoding full-length tau or tau 1–368 (Supplementary Fig. 4a,b). Hence, tau fragments that lack the C-terminal tail after the N368 residue are neurotoxic. Furthermore, inhibitors of cyclin-dependent kinase 5, glycogen synthase kinase 3 and protein kinase (roscovitine, SB216763 and H89, respectively) did not attenuate the toxic effect of tau 1–368, indicating the neurotoxic effect of tau 1–368 is not dependent on its phosphorylation (Fig. 4g).

AEP-generated tau fragments form insoluble fibrils in vitro

To investigate the effect of AEP cleavage on tau fragment propensity for filament formation, we monitored accumulation of PHFs using purified His-tagged tau recombinant proteins using a thioflavin S fluorescence assay. The tau fragment 256–368 displayed the strongest effect on self-assembly into filamentous structures, followed by the tau 1–368 and tau 256–441, and these fragments all exhibited a greater ability to form PHFs than full-length tau (Supplementary Fig. 5a). Notably, tau 1–255 and tau 369–441 fragments were unable to aggregate into PHFs, whereas the mixture of these aforementioned fragments demonstrated an increased propensity to form PHFs (Supplementary Fig. 5a). Consistent with the results of the thioflavin S assay, PHFs were observed under negative stain electron
**Figure 5** Lgmn deficiency prevents tau phosphorylation, synaptic dysfunction and memory deficits in tau P301S–transgenic mice. (a) The processing of tau in wild-type, Lgmn−/−, tau P301S–transgenic and tau P301S/Lgmn−/− mice (n = 3 mice per group). (b) Immunostaining of hippocampal and cortex neurons. Data mean ± s.e.m. of 12–17 sections from 3 mice in each group. ( **P < 0.05, ***P < 0.01, Student’s t-test). Scale bar, 50 μm. (d) Synaptic density analysis (mean ± s.e.m.; n = 6 mice per group; **P < 0.01, one-way ANOVA). (f) The ratio of paired pulses (mean ± s.e.m.; n = 6 in each group; *P < 0.05, one-way ANOVA). (h) Morris water maze analysis as distance traveled (g) and integrated distance (area under the curve (AUC); h) for WT, Lgmn−/−, tau P301S–transgenic and tau P301S/Lgmn−/− mice (mean ± s.e.m.; n = 8 mice per group; *P < 0.05, one-way ANOVA). Right, swim speed of the mice (mean ± s.e.m.; n = 8 mice per group; *P < 0.05, **P < 0.01, one-way ANOVA).}

**Knockout of AEP prevents cognitive deficits in tau P301S mice**  
To assess the physiological role of AEP in synaptic function and behavior, we bred Lgmn−/− mice with tau P301S–transgenic mice to knock out Lgmn in tau P301S–transgenic mice. The genotypes of transgenic mice were validated (Supplementary Fig. 7). Immunoreactivity for the tau N368 fragment was evident in tau P301S–transgenic mice but was not detectable in tau P301S/Lgmn−/− mice (Fig. 5a). We confirmed the absence of tau N368 in tau P301S/Lgmn−/− mice by immunohistochemistry (Fig. 5b). We found a ~30% reduction of AT8- and AT100-positive neurons in tau P301S/Lgmn−/− mice compared to tau P301S–transgenic mice both in the hippocampus and cortex (Fig. 5c and Supplementary Fig. 8). Electron microscope analysis of brain sections showed that at 6 months of age, tau P301S–transgenic mice exhibited an overt reduction in synapses compared to wild-type and Lgmn−/− mice. Notably, tau P301S/Lgmn−/− mice showed greatly ameliorated synapse loss compared to tau P301S–transgenic mice (Fig. 5d and Supplementary Fig. 9a). Gocsi staining revealed that Lgmn gene deletion prevented the loss of dendritic spines in tau P301S–transgenic mice (Supplementary Fig. 9b,c). Electrophysiological analysis found that the input/output (I/O) ratio was suppressed and the averaged field excitatory post-synaptic potential (fEPSP) slope was substantially reduced in tau P301S–transgenic mice compared to wild-type and tau P301S/Lgmn−/− mice (Supplementary Fig. 10a,b), indicating that synaptic transmission is impaired in 6-month-old tau P301S–transgenic mice but rescued by Lgmn gene deletion. The
paired-pulse ratios (induced by 100- and 200-ms intervals) were lower in tau P301S–transgenic mice but higher in wild-type and tau P301S/N255A/N368A mice (mean ± s.e.m.; n = 10 mice per group; **P < 0.01, one-way ANOVA). (i) LTP of fEPSPs in the hippocampal CA1 region, which represents the molecular basis of learning and memory, was also diminished in tau P301S–transgenic mice compared to tau P301S–transgenic mice (mean ± s.e.m.; n = 10 mice per group; *P < 0.05, one-way ANOVA). Right, the swim speed of the mice expressing tau P301S or tau P301S/N255A/N368A (mean ± s.e.m.; n = 10 mice per group; P = 0.512, one-way ANOVA).

Preservation of cognition in mice expressing uncleavable tau P301S
To confirm the effects of AEP are mediated via cleavage of tau, we injected AAVs encoding human tau P301S or tau P301S uncleavable by AEP (tau P301S/N255A/N368A) into the hippocampus of wild-type mice. Two months later, we detected similar levels of tau P301S and tau P301S/N255A/N368A in the hippocampus by immunohistochemistry and western blotting with the human tau–specific antibody HT-7 (Fig. 6a,b). The density of synapse in the hippocampus of mice injected with AAV–tau P301S was substantially less than in mice injected with an AAV–GFP control. However, the synaptic density was preserved when the mice were injected with AAV–tau P301S into the hippocampus of wild-type mice (mean ± s.e.m.; n = 6 mice per group; P < 0.05, one-way ANOVA).

Electrophysiological analysis found that the paired-pulse ratios were lower in mice injected with AAVs encoding tau P301S compared with mice injected with control virus, indicating evident presynaptic dysfunction. The paired-pulse ratios were improved in mice injected with AAV–tau P301S/N255A/N368A compared to mice injected with AAV–tau P301S (Fig. 6c). Notably, mice injected with AAV–tau P301S/N255A/N368A showed improved LTP compared to mice injected with
AβV–tau P301S (Fig. 6f). The I/O curve shows that the averaged slope of fEPSPs is smaller in mice expressing tau P301S than in those expressing tau P301S/N255A/N368A (Fig. 6g,h). These results indicate that the synaptic function is preserved in mice expressing noncleavable tau P301S.

In the Morris water maze test, tau P301S/N255A/N368A–expressing mice showed decreased latency to find the platform during the training phase and increased percentage of time in the target quadrant during the probe test compared to mice injected with AAV–tau P301S, indicating preserved cognitive function (Fig. 6i). The preservation of synaptic and cognitive function by the mutation of AEP cleavage site indicates that the cleavage of tau is required for the effects of AEP.

DISCUSSION

In the present study, we have identified that AEP acts as a physiologically protease that cleaves tau in an age-dependent manner in mouse and human brain, including in AD. AEP processes tau at both N255 and N368, inhibiting tau-mediated effects on MT assembly and axon elongation. The resultant C terminus–deleted tau fragments are prone to aggregation and strongly trigger neurodegeneration, indicating that AEP activation may induce neuronal cell death through proteolytic degradation of tau. These findings fit with the most recent report that AEP is activated in human AD brains and translates from lysosomes into the cytoplasm31. Presumably, the cytoplasmic-translocated AEP cleaves tau, leading to disruption of MT dynamics, NFT formation and neurodegeneration. We noticed that the different pathogenic processes are not necessarily associated with the same AEP-derived fragment. However, the one AEP-derived fragment that does show some of the common toxic effects is tau 1–368. Our data also support that deletion of Lgmn from tau P301S–transgenic mice substantially decreases tau hyperphosphorylation and alleviates synaptic loss and memory deficit in the mice.

Previous studies in a transgenic (tau P301S) tauropathy mouse model revealed that synapse loss and microglial activation precede the appearance of NFTs, presumably owing to the impaired transport that results from tau hyperphosphorylation32. Here, we provide convergent biochemical and cellular evidence that suggests AEP cleavage of tau also contributes to synaptic loss in tau P301S–transgenic mice. Notably, tau hyperphosphorylation does not affect tau proteolytic degradation by AEP. Thus, although tau phosphorylation may prevent its cleavage by caspase-3 (ref. 33), AEP cleavage of tau seems to be independent of phosphorylation. However, we found that truncation of tau by AEP facilitates tau hyperphosphorylation. Consistent with our findings, expression of truncated tau 151–391 in the brain of transgenic animals induces the complete tau cascade of neurofibrillary degeneration as found in humans34. Therefore, all these observations support truncation of tau as a key post-translational modification provoking neurofibrillary degeneration with pathological features similar to those found in human AD.

AEP levels increase in an age-dependent manner. As expected, tau is fragmented by AEP in mouse brain at the age of 8 months, when AEP expression is high. NFTs are the first pathology that occurs during aging and AD, and their burden correlates with the degree of cognitive impairment35–37, providing the initial circumstantial evidence to suggest that toxic gains of function by NFTs might play an important part in the progression of the disease. The reduction in the amount of hyperphosphorylated tau in tau P301S/Lgmn−/− mice compared to age-matched tau P301S–transgenic mice indicates AEP might act as a crucial protease triggering NFT pathology in AD during aging. Moreover, Lgmn knockout ameliorates synaptic loss, LTP impairment and memory loss in tau P301S–transgenic mice, supporting that AEP might be an upstream trigger in AD onset and progression. The preservation of synaptic and cognitive function in mice expressing tau P301S uncleavable by AEP further confirms that the effects of AEP are mediated by the cleavage of tau. Conceivably, blockade of this protease may provide an innovative therapeutic intervention for treating neurodegenerative diseases, including AD.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

K.Y. conceived the project, designed the experiments and wrote the manuscript. Z.Z. designed and performed most of the experiments. M.S. and S.P.Y. performed the electrophysiological experiments. X.L. prepared primary neurons and assisted with animal experiments. S.S.K. performed the stereotaxic injection of virus. D.M.D. and N.T.S. performed the mass spectrometry analysis. I.-S.K. assisted with the molecular biology experiments. L.C., W.T.H., Z.L., J.Z.-W., Y.E.S. and A.I.L. designed the experiments, assisted with data analysis and interpretation and critically read the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Tau P301S mice on a C57BL/6J background (line PS19) and wild-type C57BL/6J mice were products of the Jackson Laboratory (stock number: 008169 and 000664, respectively). The AEP knockout mice on a mixed 129/Ola and C57BL/6J background were generated as reported35. Animal care and handling was performed according to the Declaration of Helsinki and Emory Medical School guidelines. Tau P301S mice were crossed with Lgmn−/− mice. The following animal groups were analyzed: WT, Lgmn−/−, tau P301S and tau P301S/Lgmn−/−. 6-month-old male mice were used for experiments unless otherwise mentioned (8 mice per group). Sample size was determined by Power and Precision (Bistat). Investigators were blinded to the group allocation during the animal experiments. The protocol was reviewed and approved by the Emory Institutional Animal Care and Use Committee.

Human tissue samples. Post-mortem brain samples were dissected from frozen brains of 8 AD cases (age 74.5 ± 11.2 years, mean ± s.d.) and 8 non-demented controls (age 73.9 ± 12.7 years) from the Emory Alzheimer’s Disease Research Center. The study was approved by the Biospecimen Committee. AD was diagnosed according to the criteria of the Consortium to Establish a Registry for AD and the National Institute on Aging. Diagnoses were confirmed by the presence of amyloid plaques and neurofibrillary tangles in formalin-fixed tissue. Informed consent was obtained from the subjects.

Transfection and infection of the cells. HEK293 cells were transfected with plasmids encoding wild-type or point mutant mutated GST-tau isoform (4 amino acids 1–352), myc-AEP, myc-AEP C189S or myc-AEP N323A by the calcium phosphate precipitation method. To express myc-AEP, tau full-length or tau fragments in primary neurons, Lipofectamine 2000 (Invitrogen) was used as described previously39. To express tau full-length and tau 1–368 in primary neurons, 1 µl AA-tau or AA-tau 1–368 (1 x 10^14 vg ml -1) was added to 1 ml culture medium. The expression of tau full-length and tau 1–368 was assessed 96 h after infection.

In vitro tau cleavage assay. To assess the cleavage of tau by AEP in vitro, HEK293 cells were transfected with GST-tau DNA by the calcium phosphate precipitation method. 48 h after transfection, the cells were collected, washed once in PBS, lysed in buffer (50 mM sodium citrate, 5 mM DTT, 0.1% CHAPS and pH 5.5, 0.5% Triton X-100) and centrifuged for 10 min at 14,000 g at 4 °C. The supernatants were then incubated with mouse kidney lysate at pH 7.4 or 6.0 at 37 °C for 30 min. To test the effects of caspase, cathepsin, calpain, thrombin, PSA and AEP inhibitors on the cleavage of tau by AEP, inhibitors were used against caspase (ZVAD-fmk, Calbiochem), cathepsin E64, Sigma-Aldrich), calpains (ALLN, Sigma-Aldrich), thrombin (PMSF, Sigma-Aldrich), PSA (purimycin, Sigma-Aldrich) and AEP (AENK peptide inhibitor and inactive control AEKQ). To measure the cleavage of purified tau fragments by AEP, caspase-3 or calpain, GST-tagged tau full-length or fragments were purified with gluthatione beads. The purified tau protein was incubated with recombinant AEP protein (Novoprotein, 5 µg ml -1) in AEP buffer (50 mM sodium citrate, 5 mM DTT, 0.1% CHAPS and 0.5% Triton X-100, pH 6.0), recombinant caspase-3 (Calbiochem, 5 µg ml -1) in caspase buffer (100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% glycerol and 0.1% CHAPS, pH 7.4) or recombinant calpain (Sigma-Aldrich, 50 µg ml -1) in calpain buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM DTT, 1 mM EDTA, 3 mM CaCl 2, pH 7.5). The samples were then boiled in 1× SDS loading buffer and analyzed by immunoblotting.

AEP activity assay and measurement of pH in the brain lysates. Tissue homogenates or cells lysates (10 µg) were incubated in 200 µl assay buffer (20 mM citric acid, 60 mM NaH 2PO 4, 1 mM EDTA, 0.1% CHAPS and 1 mM DTT, pH 6.0) containing 20 µM AEP substrate Z-Ala-Ala-Asn-AMC ( Bachem). AMC released by substrate cleavage was quantified by measuring at 460 nm in a fluorescence plate reader at 37 °C for 1 h in kinetic mode for 5 min. The pH of hippocampus and cortex of tau P301S and control mice was read at 37 °C using Buffer A: 0.1% formic acid, 0.1% acetonitrile in water; Buffer B: 0.1% formic acid in acetonitrile) generated by a NanoAcquity UPLC system (Waters Corporation). The peptides were ionized with 2.0 kV electrospray ionization voltage from a nano-ESI source (Thermo) on a hybrid LTQ XL Orbitrap mass spectrometer (Thermo). Data-dependent acquisition of centroid MS spectra at 30,000 resolution and MS/MS spectra were obtained in the LTQ following collision-induced dissociation (collision energy 35%, activation Q 0.25, activation time 30 ms) for the top 10 precursor ions, with charge determined by the acquisition software to be z ≥ 2. Dynamic exclusion of peaks already sequenced was for 20 s with early expiration for 2 count events with signal-to-noise > 2. Automatic gating control was set to 150 ms maximum injection time, or 10° counts. To identify AEP cleavage sites on human tau, the SageN Sorcerer SEQUEST 3.5 algorithm was used to search and match MS/MS spectra to a complete semi-trypsin human proteome database (NCBI reference sequence revision 50, with 66,652 entries) plus pseudo-reversed decoys sequences41,42 with a 20 p.p.m. mass accuracy threshold. Only b and y ions were considered for scoring (Xcorr) and Xcorr values are averaged for increased groups of peptides organized by a combination of trypticity (fully or partial) and precursor ion charge state to remove false-positive hits along with decoys until achieving a false discovery rate (FDR) of <5% (<0.25% for peptides identified by more than one peptide). The FDR was estimated by the number of decoy matches (nd) and total number of assigned matches (nt). FDR = nd/nt, assuming mismatches in the original database were the same as in the decoy database. All semi-trypsin MS/MS spectra for putative AEP generated APP cleavage sites were manually inspected. A user-defined precursor mass tolerance of ± 20 p.p.m. was employed for extracted ion chromatogram (XIC)-based quantification as previously described42.

Western blot analysis. Mouse brain tissue or human tissue samples were lysed in lysis buffer (50 mM Tris, pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1.5 mM Na 3VO 4, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, supplemented with protease inhibitors cocktail), and centrifuged for 15 min at 16,000 g. The supernatant was boiled in SDS loading buffer. After SDS-PAGE, the samples were transferred to a nitrocellulose membrane. Primary antibodies to the following targets were used: GST-HRP (Sigma-Aldrich, GERP1236, 1:1,000), tubulin (Sigma-Aldrich, T6074, 1:5,000), HA (Santa Cruz, sc-7392, 1:1,000), myc (Calbiochem, OP10, 1:1,000), His (GE Healthcare, 27-4710-01, 1:2,000), H777 (Thermo, MN1000, 1:500), tau5 (Thermo, MA3-12805, 1:1,000), AT8 (Thermo, MN1020, 1:1,000) and AT100 (Thermo, MN1060, 1:1,000), and tau-1 (Calbiochem, MAB3420, 1:5,000).

Immunostaining. For visualization of PHF-tau, free-floating 30-µm brain sections were treated with 0.3% H 2O 2 for 10 min. Then sections were washed three times in PBS and blocked in 1% BSA, 0.3% Triton X-100, for 30 min followed by overnight incubation with anti-tau N368 (generated and verified as described in the main text, 1:1,000), AT8 (Thermo, MN1020, 1:500) or AT100 antibody (Thermo, MN1060, 1:500) at 4 °C. The signal was developed using Histostain-SP kit (Invitrogen). To detect the localization of AEP-cleaved tau and PHF in human AD brain sections, the sections were incubated with mouse anti-tau N368 primary antibody overnight at 4 °C. The sections were washed three times in PBS and incubated with Texas Red-conjugated anti-mouse IgG for 1 h at room temperature. After brief rinse in PBS, the sections were stained for 5 min with 0.0125% thiolflavin-S in 50% ethanol. The sections were washed with 50% ethanol and placed in distilled water. Then, the sections were covered with a glass cover using mounting solution and examined under a fluorescence microscope.

Purification of recombinant tau protein and preparation of PHF. His-tagged full-length tau and tau 1–255, 1–368, 256–368, 256–441 and 368–441 were purified from E. coli using his bind purification kit (Calbiochem). The purified tau fragments were induced to aggregate as described previously41. Briefly, purified tau fragments (50 µM) were incubated in PBS (pH 7.4) containing 12.5 µM heparin, 2 mM DTT and a protease inhibitor cocktail. The samples were incubated with 50 µM thioflavin S for 45 min in the dark at room temperature. PHF

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formation was quantified by measuring the fluorescence with an excitation at 440 nm and an emission wavelength of 510 nm. The overall appearance of the PHFs was visualized by negative stain electron microscopy. Briefly, the reaction samples was adsorbed onto carbon/formvar-coated 400 mesh copper grids (EM Sciences) for 30 s and stained with 2% uranyl acetate for 30 s. Excess liquid in the sample was wicked using filter paper. The grids were examined with a Philips 208S electron microscope (Philips, Hillsboro, OR). The remaining solutions of aggregated tau were centrifuged at 100,000g for 30 min to separate aggregated tau pellet and non-aggregated tau supernatant and analyzed by western blot.

Microtubule assembly assay. His-tagged full-length tau and tau fragments were purified from E. coli. 1.5 µM tau fragments were incubated with 20 µM tubulin (Cytoskeleton) at 37 °C, and polymerization of microtubules was monitored by measuring the absorbance at 350 nm over 20 min as described previously44.

AAV vector packaging. AAV vectors carrying the longest human tau isoform (Tau4R2N) or GFP use the human synapsin1 promoter to drive neuron-specific gene expression45. All of the mutations were introduced using site-directed mutagenesis kit (Agilent Technologies). The AAV particles were prepared by Viral Vector Core at Emory University.

Stereotaxic injection of the virus. 3-month-old wild-type C57BL/6J mice were anesthetized with phenobarbital (75 mg kg−1). Bilateral intracerebral injection of AAV–GFAP–AAV–tau P301S and AAV–tau P301S/N255A/N368A was performed stereotactically at coordinates posterior 1.94 mm, lateral 1.4 mm, ventral 2.1 mm relative to bregma. 2 µl of viral suspension containing 2 × 1011 vector genome (vg) was injected in to each point using 10-µl glass syringes with a fixed needle at a rate of 0.5 µl min−1. The needle remained in place for 5 min before it was removed slowly (throughout 2 min). The mice were placed on a heating pad until they began to recover from the surgery.

Electron microscopy of synapse. Synaptic density was determined by electron microscopy. Briefly, after deep anesthesia, mice were perfused transcardially with 2% glutaraldehyde and 3% paraformaldehyde in PBS. Hippocampal slices were postfixed in cold 1% OsO4 for 1 h. Samples were prepared and examined using 2% glutaraldehyde and 3% paraformaldehyde in PBS. Hippocampal slices were microtome-cut at 30 µm, immersed in 3% potassium bichromate for 3 d in the dark. The solution was renewed slowly (throughout 2 min). The mice were placed on a heating pad until they began to recover from the surgery.

Generation of antibodies that specifically recognize the AEP-generated tau fragment (anti–tau N368). Three Balb/c mice were immunized with the peptide Ac-CITHVGPPGGN-OH, which includes the 9 amino acids in tau that precede the AEP cleavage site at N368 as well as an amino-terminal cysteine residue to allow coupling to KLH. The mice were boosted 4 times with the immunizing peptide with 3-week intervals between injections. The antisera was pooled and the titers against the immunizing peptide were determined by ELISA. The maximal dilution giving a positive response using chromogenic substrate for horseradish peroxidase was 1:30,000. The immunoreactivity of the antisera was further confirmed by western blot and immunohistochemistry.

Morris water maze. 6-month-old WT, Lgmn−/−, tau P301S and tau P301S/ Lgmn−/− mice were trained in a round, water-filled tub (52 inch diameter) in an environment rich with extra maze cues as described previously44. Each subject was given 4 trials/day for 5 consecutive days with a 15-min intertrial interval. The maximum trial length was 60 s, and if subjects did not reach the platform in the allotted time, they were manually guided to it. Following the 5 d of task acquisition, a probe trial was presented, during which time the platform was removed and the percentage of time spent in the quadrant that previously contained the escape platform during task acquisition was measured over 60 s. All trials were analyzed for latency and swim speed by means of MazeScan (Clever Sys, Inc.).

Fear conditioning test. The ability to form and retain an association between an aversive experience and environmental cues was tested with a standard fear conditioning paradigm that occurs over a period of 3 d. Mice were placed in the fear conditioning apparatus (7” W, 7” D x 12” H, Coulbourn) composed of Plexiglass with a metal shock grid floor and allowed to explore the enclosure for 3 min. Following this habituation period, 3 conditioned stimulus (CS)–unconditioned stimulus (US) pairings were presented with a 1-min intertrial interval. The CS was composed of a 20-s, 85-dB tone and US was composed of 2 s of a 0.5–mA footshock, which was co-terminate with each CS presentation. One minute following the last CS–US presentation, mice were returned to their home cage. On day 2, the mice were presented with a context test, during which subjects were placed in the same chamber used during conditioning on day 1 and the amount of freezing was recorded via a camera and the software provided by Coulbourn. No shocks were given during the context test. On day 3, a tone test was presented, during which time subjects were exposed to the CS in a novel compartment. Initially, animals were allowed to explore the novel context for 2 min. Then the 85-dB tone was presented for 6 min and the amount of freezing behavior was recorded.

Primary neuron cultures. Primary rat cortical neurons were cultured as previously described26. The rats were brought from the Jackson Laboratory. The protocol was reviewed and approved by the Emory Institutional Animal Care and Use Committee. To measure the effect of tau fragments on neurons, neurons cultured 7 d in vitro (DIV 7) were transfected with myc-AEP HA-tau full-length, or HA-tau fragments using Lipofectamine 2000 (Invitrogen). 72 h later, the neurons were fixed in 4% formaldehyde, permeabilized and immunostained with anti–MAP2 (Sigma-Aldrich, M9942, 1: 1,000), anti-myc (Calbiochem, OP10, 1:1,000), anti-HA (Santa Cruz, sc-7392, 1:1,000), AT8 (Thermo, MN1020, 1:1,000), anti-MAP2 (Sigma-Aldrich, M9942, 1: 1,000), anti-myc (Calbiochem, OP10, 1:1,000), anti-HA (Santa Cruz, sc-7392, 1:1,000), AT8 (Thermo, MN1020, 1:500), AT100 (Thermo, MN1060, 1:500) or anti-tau N368 (generated and verified as described in the main text, 1:1,000) antibody. The toxic effect of tau fragments was detected with the In situ cell death detection kit (Roche). The apoptotic index was expressed as the percentage of TUNEL-positive neurons out of the total number of MAP2-positive neurons.

Caspase and cathepsin activity assay. Cell lysates (10 µg) were incubated in 200 µl caspase assay buffer (100 mM HEPS 0.1% CHAPS, 10% sucrose, pH 7.4) containing 25 µM caspase substrate Ac-Asp-Glu-Val-Asp-AMC (Bachem), or in 200 µl cathepsin assay buffer (100 mM sodium acetate, 1 mM EDTA, pH 5.5) containing 25 µM cathepsin substrate D-Val-Leu-Lys-AMC (Bachem) for 1 h. AMC released by substrate cleavage was quantified by measuring at 460 nm in a fluorescence plate reader.
Statistical analyses. Statistical analyses were performed using either Student’s t-test (two-group comparison) or one-way ANOVA followed by LSD post hoc test (more than two groups), and differences with P values less than 0.05 were considered significant.

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