Delta-secretase cleaves amyloid precursor protein and regulates the pathogenesis in Alzheimer's disease

Zhentao Zhang, Emory University
Mingke Song, Emory University
Xia Liu, Emory University
Seong Su Kang, Emory University
Duc M. Duong, Emory University
Nicholas Seyfried, Emory University
Xuebing Cao, Huazhong University of Science and Technology
Liming Cheng, Tongji University School of Medicine
Yi E. Sun, Tongji University School of Medicine
Shan Ping Yu, Emory University

Only first 10 authors above; see publication for full author list.

Journal Title: Nature Communications
Volume: Volume 6
Publisher: Nature Publishing Group: Nature Communications | 2015-11-01, Pages 8762-8762
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1038/ncomms9762
Permanent URL: https://pid.emory.edu/ark:/25593/rghp1

Final published version: http://dx.doi.org/10.1038/ncomms9762

Copyright information:
© 2015 Macmillan Publishers Limited.

This is an Open Access work distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).
Delta-secretase cleaves amyloid precursor protein and regulates the pathogenesis in Alzheimer’s disease

Zhentao Zhang¹,², Mingke Song³, Xia Liu¹, Seong Su Kang¹, Duc M. Duong⁴,⁵, Nicholas T. Seyfried⁴,⁵, Xuebing Cao⁶, Liming Cheng⁷, Yi E. Sun⁷,⁸, Shan Ping Yu³, Jianping Jia⁹, Allan I. Levey⁴ & Keqiang Ye¹

The age-dependent deposition of amyloid-β peptides, derived from amyloid precursor protein (APP), is a neuropathological hallmark of Alzheimer’s disease (AD). Despite age being the greatest risk factor for AD, the molecular mechanisms linking ageing to APP processing are unknown. Here we show that asparagine endopeptidase (AEP), a pH-controlled cysteine proteinase, is activated during ageing and mediates APP proteolytic processing. AEP cleaves APP at N373 and N585 residues, selectively influencing the amyloidogenic fragmentation of APP. AEP is activated in normal mice in an age-dependent manner, and is strongly activated in 5XFAD transgenic mouse model and human AD brains. Deletion of AEP from 5XFAD or APP/PS1 mice decreases senile plaque formation, ameliorates synapse loss, elevates long-term potentiation and protects memory. Blockade of APP cleavage by AEP in mice alleviates pathological and behavioural deficits. Thus, AEP acts as a δ-secretase, contributing to the age-dependent pathogenic mechanisms in AD.
Amyloid β (Aβ) is a key pathogenic factor in Alzheimer’s disease (AD). It aggregates to form neurotoxic oligomers and insoluble deposits in plaques in the brains of affected individuals, contributing to the progressive loss of synaptic function and cognitive impairment. Aβ peptides are derived from the sequential proteolytic processing of amyloid precursor protein (APP) by a group of proteases. APP is a type I oriented membrane protein with its amino terminus within the lumen/extracellular space and its carboxyl terminus within the cytosol. Three proteases, α-, β-, and γ-secretases, regulate APP processing. Commitment of APP into amyloidogenic and non-amyloidogenic processing depends on the cellular levels of α- and β-secretases and the traffic of APP to subcellular organelles expressing these proteases. The amyloidogenic pathway leads to Aβ generation, while the anti-amyloidogenic pathway prevents Aβ generation.

β-Secretase activity mediates the initial and rate-limiting processing step leading to Aβ generation. β-Secretase (BACE1) is a membrane-bound aspartyl protease with its active site in the lumen/extracellular space and an acidic pH optimum (around pH 4.5; ref. 8), consistent with the major site of β-secretase activity within endosomes. The β-secretase-cleaved C-terminal portion of APP is subsequently processed by γ-secretase to liberate Aβ. Mounting evidence shows that γ-secretase cleaves APP in the endosomal/lysosomal system, including phagosomes and autophagosomes. The protease activity of γ-secretase is also regulated by pH with optimal activity at pH 6.3 (ref. 10). An alternative pathway of anti-amyloidogenic processing of APP occurs within the Aβ domain between residues K612 and L613 in APP1-12 and results in the secretion of the large APP amino-terminal domain and the generation of α-CTF (C83). This cleavage is performed by α-secretases, which in neurons includes ADAM10 (ref. 13). α-Secretase processing of APP occurs in the trans-Golgi network and the plasma membrane. On the basis of the relative BACE1 expression levels, neurons have been suggested to be the major source of Aβ produced in the brain.

Mammalian asparagine endopeptidase (AEP), also known as legumain, is a widely distributed lysosomal cysteine protease that cleaves after asparagine residues. AEP activation is autocalytic and requires sequential removal of C- and N-terminal propeptides at different pH thresholds. Disruption of AEP leads to late endosome and lysosome abnormalities with accumulation of electron-dense and/or membranous material. AEP has diverse physiological functions in the brain. Neuronal AEP is activated by acidosis during excitotoxicity and contributes to neuronal apoptosis by degrading the DNase inhibitor SET, a binding partner of APP19,20. AEP cleavage of SET (I5P2A) inhibits PP2A and upregulates tau hyperphosphorylation. AEP also cleaves TDP-43 in human cases of frontotemporal lobar degeneration. Moreover, AEP cleaves tau, mediating neurofibrillary pathology in AD. AEP is inhibited by cystatin C, a secreted protein found in human cerebrospinal fluid (CSF) that binds soluble Aβ and inhibits its oligomerization, and which is reduced in AD. Cystatin C interacts with active AEP in the lysosome and acts as an endogenous inhibitor of AEP. Together, these observations led us to hypothesize that AEP plays an important role in AD pathogenesis.

In this report, we show that AEP is a physiological substrate of AEP. AEP increases with ageing and proteolytically cleaves APP in the ectodomain, affecting the rate of BACE1 cleavage of the resultant substrate. Knockout of AEP in vivo using 5XFAD and APP/PS1 mouse models of AD reduces Aβ burden, improves synapse integrity and preserves memory. Blockade of APP cleavage by AEP prevents pathological and behavioural defects induced by overexpression of APP. These findings suggest that AEP is activated during ageing and promotes Aβ production, contributing to AD onset and progression. Hence, AEP may act as a novel δ-secretase that mediates APP fragmentation and amyloidogenesis.

**Results**

APP is a physiological substrate of AEP. To investigate whether APP is a substrate of AEP, we prepared kidney lysates derived from wild-type (WT; +/+) or AEP knockout (−/−) mice at different pH to inactivate (pH 7.4) or activate AEP (pH 6.0), respectively, and incubated with recombinant green fluorescent protein (GFP)-APP. Immunoblotting analysis revealed robust APP cleavage with two major fragments in WT samples under pH 6.0 but not pH 7.4. By contrast, we failed to observe any APP cleavage in AEP-null samples regardless of pH values. We also validated the APP fragmentation with anti-APP N-terminal antibody (Fig. 1a, left panels), and confirmed the pH dependence of AEP enzyme activity in WT samples (Fig. 1a, right panel). We used inhibitory anti-AEP antibodies to verify the role of AEP. As anti-AEP doses gradually increased, APP fragmentation was progressively inhibited and completely blocked at 50 μg ml−1. In contrast, the same concentrations of anti-mouse IgG failed to block APP cleavage (Fig. 1b). Mutation of either of two key residues in AEP blinded its proteolytic activity against APP (Fig. 1c), including C189 that is essential for the cysteine protease activity and N323 that plays a critical role in the cleavage and maturation of AEP17,19. In addition, the selective AEP inhibitor peptide AENK suppressed APP cleavage, while an inactive peptide AEKQ had no effect. AEP enzymatic activity tightly correlated with the APP shedding pattern (Fig. 1d). We also explored whether or not endogenous APP in mouse brain can be processed by AEP. Endogenous APP was robustly cleaved in WT (AEP+/+) mouse brain lysates with acidic conditions that activate AEP, whereas the cleavage was abrogated at pH 7.4 where APP is catalytically inactive. APP cleavage was also absent in brain lysates from AEP knockout mice (AEP−/−; Fig. 1e). To ascertain if AEP is indeed a direct substrate of AEP, we incubated purified glutathione S-transferases (GST)-APP with active recombinant human AEP. AEP potently cleaved GST-APP recombinant proteins with the same pattern as in the tissues (Fig. 1f). Thus, the biochemical cleavage assay, antibody or peptide inhibition assay and studies with AEP knockout tissues all indicate that APP is a physiological substrate of AEP.

AEP cleaves APP at N373 and N585 residues. To identify potential AEP cleavage sites on APP, we generated a series of truncated APP proteins with sequential deletions of N-terminal domains (Supplementary Fig. 1) and tested them in the biochemical cleavage assay. Deletion of the N terminus proximal to amino acid 289 did not affect APP cleavage (Supplementary Fig. 1), indicating that AEP cleaves APP after residue 289. Next, we purified the cleaved recombinant protein products and analysed them by mass spectrometry (MS), identifying two main cleavage sites at N585 and N373 that yield fragments of molecular weight ~130 and 80 kDa, respectively (Fig. 2a). The amino acid number is based on the APP695 isoform. To determine if the same APP cleavages occur in human brain from AD cases, we immunoprecipitated brain lysates with anti-APP N-terminal antibody and subjected the samples to proteomic analysis. An APP fragment terminated at N585 was detected (Fig. 2b). Mutations of N373A or N585A abolished the 80- or 130-kDa fragments, respectively, and the APP double mutant (N373A/N585A) was resistant to both cleavages. By contrast, mutations at other sites (N391A, N400A or N417A) had no effect on APP fragmentation (Fig. 2c). Thus, these studies suggest that N373 and N585 are the two major AEP cleavage sites in APP.
Figure 1 | AEP cleaves APP in vitro. (a) APP cleavage by AEP⁺⁺/⁺⁺ kidney lysates. APP was cleaved at pH 6.0 by AEP⁺⁺/⁺⁺ kidney lysates (left panels). The enzymatic activity of AEP was determined using AEP activity assay (right panel; mean ± s.e.m. of three independent experiments). (b) Antibody titration assay. Kidney lysates were incubated with AEP-specific antibody for 5 min, and then incubated with GST-APP for 30 min. The processing of APP was determined using western blot. (c) Mutants of AEP C189 and N323 diminish APP cleavage. (d) Blocked of APP cleavage by Fmoc-Ala-Glu-Asn-Lys-NH₂ (AENK) peptide (left panel). AEP activity was inhibited by AENK but not by Fmoc-Ala-Glu-Gln-Lys-NH₂ (AEQK) (right panel; mean ± s.e.m.; n = 3). (e) Cleavage of endogenous APP by AEP. (f) Western blot showing the processing of purified GST-APP by recombinant AEP. The AEP-derived APP fragments were detected using anti-APP N-terminal antibody (left panel) and anti-APP C-terminal antibody (right panel). a.f.u., arbitrary fluorescence unit; DMSO, dimethylsulfoxide; MW, molecular weight; WB, western blot.
**Figure 2 | AEP cleaves APP at N373 and N585 residues.** (a) Proteomic analysis of APP recombinant proteins processed by AEP. The detected peptide sequences indicate that N585 and N373 are the two main cleavage sites with the shed bands of molecular weight (MW) ~130 and 80 kDa, respectively. (b) Proteomic analysis of APP fragments in AD brain. APP fragments cleaved after N585 was detected. (c) Processing of various mutant APP by AEP.

**AEP regulates Aβ production.** APP can be processed by several proteases, with both α- and β-secretases shedding APP ectodomains (Fig. 3a). To determine if AEP processing of APP affects α- and β-secretase cleavages, recombinant APP proteins corresponding to the two AEP-processed derivatives (APP374–695 and APP386–695) were tested as substrates for ADAM10 and BACE1 protease, respectively. BACE1 processed both full-length APP and the truncated APP374–695 fragment at the same rate. Interestingly, BACE1 processing of the APP386–695 fragment yielded more C99 product than full-length APP or APP374–695 (Fig. 3b). By contrast, ADAM10 processed all these proteins at the same rate (Fig. 3c), indicating that APP386–695 might be a better substrate for BACE1 than full-length APP or APP374–695. To further test this possibility, we incubated purified GST-APP386–695 with BACE1 in the presence or absence of AEP-generated APP N-terminal fragment APP1–373 or APP1–585, respectively. APP1–585 but not APP1–373 decreased C99 production in a concentration-dependent manner (Supplementary Fig. 2a). However, AEP-generated APP N-terminal fragments had no effect on ADAM10 cleavage of APP386–695 (Supplementary Fig. 2b). These data suggest that the N terminus of APP1–585 might inhibit BACE1 processing of the resultant cleavage product APP386–695. Conceivably, cleavage of APP by AEP at N585 may relieve the steric hindrance of the N terminus and thereby accelerate APP processing by BACE1. Since BACE1 is the rate-limiting enzyme for Aβ production, we also explored the role of AEP in Aβ production in neurons. Remarkably, while total APP protein levels were similar between WT and AEP−/− neurons, the concentrations of Aβ40 and Aβ42 in the conditioned medium of AEP−/− cultures were significantly lower than those in AEP+/+ cultures. The concentration of sAPPβ in the AEP−/− conditioned medium was higher than that of AEP+/+ cultures (Fig. 3d). Though AEP is assumed to be highly expressed in microglia29, we found it is abundant in primary neuronal cultures as well. The percentage of neurons was about 96% in both AEP+/+ and AEP−/− neuronal cultures (Supplementary Fig. 2c). To further confirm these results, we transfected HEK293 cells stably expressing human APP with two different AEP siRNAs or control siRNA. Depletion of AEP in these cells significantly reduced Aβ production and increased sAPPβ levels without altering the level of total APP (Fig. 3e).
Conversely, overexpression of the APP<sub>586–695</sub> fragment markedly elevated Aβ production compared with overexpression of full-length APP and the APP<sub>374–695</sub> fragment. To further evaluate the substrate preference of BACE1 for the various forms of APP, we also carried out GST pull-down assays, and found that the APP<sub>586–695</sub> fragment bound more BACE1 than full-length APP and APP<sub>374–695</sub> (Fig. 3f). Furthermore, when the APP<sub>586–695</sub> fragment was co-expressed in HEK293 cells with increasing...
amounts of the myc-APP1–585 or myc-APP1–373, the longer
APP1–585 fragment selectively decreased the production of Aβ in
a dose-dependent manner and reduced the binding of BACE1
to APP586–695 (Supplementary Fig. 2d). Accordingly, APP mutation
analysis revealed Aβ production was reduced by inhibition of
AEP cleavage of APP at N585, but not at N373. Since cell surface
trafficking of APP affects its processing, we tested the possibility
that these mutations affect the internalization of cell surface APP.
Cell surface biotinylation assays demonstrated that neither the
N585 nor the N373 mutations had any effect on APP
internalization (Fig. 3g). Hence, AEP cleavage of APP at N585
facilitates the subsequent processing by BACE1 and generation of
Aβ.

Since AEP pre-processing of APP directly influences subse-
quently cleavage by BACE1, we sought to determine if other
proteases might affect AEP cleavage of APP. APP is processed by
caspase-3 and cathepsin in addition to the secretases. We
employed pharmacological inhibitors of these proteases and
found that only the AEP inhibitory peptide AENK antagonized
the secretases. Other small molecular inhibitors and inactive
peptide AEQK were without effect (Supplementary
Fig. 3a). Moreover, APP point mutants that influence cleavage
were fractionated by differential centrifugation on a sucrose
environment and associated with cell surface. We
employed pharmacological inhibitors of these proteases and
found that only the AEP inhibitory peptide AENK antagonized
APP processing by AEP, whereas other small molecular inhibitors
and inactive peptide AEQK were without effect (Supplementary
Fig. 3a). Moreover, APP point mutants that influence cleavage
by a variety of secretases were strongly cleaved by AEP
(Supplementary Fig. 3b), suggesting that APP cleavage by
secretases does not affect AEP processing of its substrate APP.

To further define the role of BACE1, γ-secretase or ζ-secretase
in AEP processing of APP, we also used BACE1−/−, PS1/PS2−/−
mouse embryonic fibroblast cells. Proteolytic analysis revealed that AEP
initiated APP fragmentation in a time-dependent manner with comparable rates regardless of the
presence or absence of BACE1, γ-secretase or ζ-secretase
(Supplementary Fig. 3c). Therefore, these studies demonstrate
that the secretases do not interfere with AEP’s cleavage of APP.

AEP interacts with APP in the endolysosomal organelles.
To explore how a lysosomal protease AEP cleaves APP and leads to
secretion of the relevant APP fragments, we investigated the
subcellular basis for the interaction between AEP and APP in
brain. Brain samples from WT, 5XFAD and tau P301S mice
were fractionated by differential centrifugation on a sucrose
discontinuous gradient to separate cellular organelles. For the
WT brain samples, AEP was enriched in fractions 9 and 10,
containing the specific lysosomal marker LAMP1, and APP was
enriched in fractions 4–6, co-enriched with EEA1, a specific
marker for the endosome, and GGA3, a Golgi-localized adapter
protein involved in BACE1 trafficking. In the aged WT mouse brain, 5XFAD and tau P301S transgenic mouse brain, AEP
distribution was more widely distributed in fractions 5–12,
consistent with its upregulation during ageing and in AD (Fig. 6).
Moreover, AEP overlapped with APP in fractions 5 and 6
together with the endolysosomal markers LAMP1 and EEA1.
BACE1 was highly enriched in the same fractions (Fig. 4b and
Supplementary Fig. 4a,b). To detect the APP fragments derived
from AEP processing in the fractionated brain samples, we
developed cleavage-site-specific antibodies, which selectively bind
the following AEP-derived fragments: APP1–373 (anti-APP 373N),
APP1–585 (anti-APP 585N) and APP586–695 (anti-APP 585C). The
specificity of these antibodies was confirmed by western blot and
immunohistochemistry (Supplementary Fig. 5a–c). APP1–373 and
APP1–585 fragments were enriched in fractions 5 and 6 of 5XFAD
mice. Thus, these data suggest that AEP cleaves APP in the
endolysosomal system. Accordingly, the cleavage of APP by AEP
in endolysosomes may promote the subsequent BACE1 cleavage
and production of Aβ.

Aβ exerts a variety of deleterious effects on cells including
altering membrane protein traffic. To explore the effects of Aβ
on the membranous compartments where AEP may cleave APP,
we treated primary cortical neurons with different doses of
pre-aggregated Aβ for 24 h and determined the subcellular
distribution of APP and AEP by immunofluorescent staining.
Remarkably, Aβ (20 μM) treatment triggered the co-localization
of APP and AEP in primary neurons (Fig. 4c, left panels
and Supplementary Fig. 6a). APP also co-localized with
the endosomal marker EEA1 (Fig. 4c, right panels and
Supplementary Fig. 6b). The co-localization of AEP with EEA1
and LAMP1 in Aβ-treated neurons and 5XFAD mice brain
was confirmed by confocal microscopy (Supplementary Fig. 6c–i).
Notably, Aβ treatment elicited AEP expression and activation
(Supplementary Fig. 7a,d). Using an internalization assay, we found that Aβ
promoted the endocytosis of APP (Fig. 4e). Moreover, APP and AEP were co-immunoprecipitated from both WT and
5XFAD brain lysates, confirming their interaction (Fig. 4f).

AEP is secreted extracellularly in the tumour micro-
environment and associated with cell surface. We
found that AEP was secreted into the CSF, and interestingly, the
AEP-cleaved APP1–585 fragment was significantly higher in
human AD CSF than in healthy controls (Supplementary
Fig. 7a,b). Furthermore, exogenous His-tagged AEP recombinant proteins bound to APP on the cell surface, and
were internalized and subsequently processed to the active
form (Supplementary Fig. 7c,d). Thus, AEP interacts with APP on the
cell surface, and the complex may be endocytosed. To study how Aβ
peptides are secreted from the endolysosome system, we
generated transgenic mice overexpressing APP, with tetanus toxin, a well-established inhibitor of exocytosis.
Inhibition of exocytosis increased the Aβ levels in the cell body
(Supplementary Fig. 7e), indicating that Aβ may be exocytosed,
fitting with previous observations. Hence, our data support that
AEP interacts with APP and proteolytically processes it in the
endolysosomal organelles.

APP N-terminal ectodomain shed by AEP cleavage is neurotoxic.
Accumulating evidence indicates that the ectodomain of APP
cleavage may be tied to neurodegeneration in AD. We next
assessed whether the AEP-derived APP fragments influence
neuronal viability. strikingly, treatment of primary neurons
with purified recombinant His-tagged APP1–373, mimicking the
AEP-derived secreted APP fragment, triggered extensive axonal
fragmentation and neuronal cell death. In contrast, sAPPα,
sAPPβ, APP1–585 and APP374–585 fragments did not exhibit any
manifest neurotoxic effect (Fig. 5a–c). TUNEL staining
showed that the N-terminal APP1–373 fragment (10 μg ml−1)
induced apoptosis (Fig. 5d,e). The APP1–373 fragment was not
toxic to either PC12 cells or HEK293 cells (Fig. 5f,g), suggesting
the toxic effect is specific to certain cell types, especially neurons.
However, the AEP-derived APP fragments did not induce
neuronal apoptosis when expressed within neurons (Fig. 5h),
suggesting that only secreted N-terminal APP1–373 fragment is
neurotoxic, presumably by binding to cell surface receptors.
As shown earlier, the AEP cleavage of APP at N585 accelerates
BACE1 processing of the resultant APP586–695 fragment. Hence,
the combined effects of the two AEP cleavages of APP, leading to
increased levels of the neurotoxic secreted APP1–373 and Aβ
products, respectively, would be deleterious in AD.

AEP is upregulated and activated during ageing and in AD.
The greatest known risk factor for AD is increasing age. We
assessed AEP activity in ageing brain. AEP protein levels were
barely detectable in the brain at 2 or 3 months of age, but
escalated at 4 months and continued to increase substantially
with age. AEP enzymatic activity is dependent on an
Figure 4 | AEP interacts with APP in the endolysosomal system. (a,b) AEP and APP distribution in the subcellular fractions. Brain samples from 5-month-old WT (a) and age-matched 5XFAD (b) mice were homogenated and fractionated on a discontinuous sucrose gradient. The fractions were analysed by western blotting (WB) for AEP, APP fragments, BACE1, EEA1 (endosome marker), GGA3 (trans-Golgi network marker) and LAMP1 (lysosome marker). The relative amount of AEP, EEA1 and LAMP1 in each fraction was quantified (mean ± s.e.m. of three independent experiments, t-test, *P < 0.01 compared with WT mice). (c) APP co-localizes with AEP and EEA1. Primary neuronal cultures (DIV 12) were treated with 2 or 20 μM of pre-aggregated Aβ for 24 h, followed by immunostaining with various antibodies including anti-AEP, anti-APP or anti-EEA1. Aβ treatment increased the co-localization of APP and AEP in primary cortical neurons (left panels). APP co-localized with the endosomal marker EEA1 as well (right panels). Shown are the representative figures of two independent experiments. Scale bar, 10 μm. (d) Aβ treatment elicits AEP activation in neurons in a dose-dependent manner. (e) Internalization assay showing the effect of 20 μM Aβ on APP endocytosis. (f) Co-immunoprecipitation of APP and AEP in WT and 5XFAD mouse brain. AEP in mouse brain lysates was immunoprecipitated with anti-AEP antibody and analysed by immunoblotting with anti-APP antibody. MW, molecular weight.
Intraneuronal AEP-derived APP 586–695 immunoactivity was increased in brains from both the 5XFAD mouse model of AD and in human AD brains than control brains (Fig. 6d). Enzymatic analysis matched controls. Active AEP fragments were also increased in brains, AEP-derived APP fragments APP 1–373, APP1–585 and APP 374–585 were all increased in AD cases compared with age-matched controls. Knockout of AEP reverses synaptic dysfunction in 5XFAD mice. Although RNA-seq data suggest that AEP/LGMN is highly expressed in microglia39, immunohistochemistry staining shows that AEP immunoreactivity is also clearly enriched in neurons (Supplementary Fig. 8c). AEP and APP 586–695 are also highly expressed in neurons of 5XFAD mouse brain as confirmed by confocal microscopy (Supplementary Fig. 8d,e). To investigate the relationship between the concentrations of AEP-derived APP fragments and Aβ, we developed an enzyme-linked immunosorbent assay (ELISA) using our cleavage-stie-specific antibodies and found that the concentrations of AEP-derived APP586–695 correlated with Aβ in AD and age-matched control brains (Supplementary Fig. 8f). Collectively, these findings indicate that AEP protein expression, autocleavage and enzyme activity are all upregulated during ageing, contributing to this novel APP processing event and increased Aβ production.
the synapse loss (Fig. 7a and Supplementary Fig. 10a). We also assessed the density of dendritic spines along individual Golgi-stained pyramidal neurons. Spine density was decreased in 5XFAD mice model, and this defect was reversed in 5XFAD/AEP+/− mice (Fig. 7b and Supplementary Fig. 10b). 5XFAD mice display significantly impaired long-term potentiation (LTP) at Schaffer collateral-CA1 pathways39. Electrophysiological analysis demonstrated significantly increased LTP magnitude in 5XFAD/AEP+/− mice when compared with 5XFAD mice. In contrast, LTP was comparable between the age-matched WT and AEP+/− mice (Fig. 7c). Hence, these results show that inactivation of AEP rescues synaptic loss and LTP deficits in 5XFAD mice.

Figure 6 | AEP is upregulated and cleaves APP during ageing and in AD. (a) Western blot (WB) showing AEP and APP expressing and processing in mouse brain during ageing. (b) AEP enzymatic activity analysis (mean ± s.e.m.; n = 6; one-way analysis of variance (ANOVA), *P < 0.01 compared with 2-, 3- and 4-month-old mouse brain; **P < 0.01 compared with 2-, 3-, 4- and 6-month-old mouse brain). (c) WB showing APP fragments in AEP+/− mice brain. (d) WB detection of APP processing in human brain samples from AD patients and age-matched controls. (e) AEP activity in 6-month-old 5XFAD mice and non-transgenic controls (mean ± s.e.m.; n = 6; t-test, *P < 0.01). (f) AEP activity in human brain samples from AD patients and age-matched controls (mean ± s.e.m.; n = 6; t-test, *P < 0.01). (g) Immunostaining showing the presence of AEP-derived APP fragments in AD brain (mean ± s.e.m.; t-test, n = 8; *P < 0.01). Scale bar, 50 μm. Data were analysed using one-way ANOVA followed by post hoc comparison.
Figure 7 | AEP gene deficiency ameliorates synaptic dysfunction, Aβ deposition and cognitive deficits in the 5XFAD mouse model. (a) The density of synapse determined by electron microscopy (mean ± s.e.m.; n=6; *P<0.01). (b) Spine density in the hippocampus determined by Golgi staining (mean ± s.e.m.; n=4; *P<0.01). (c) AEP deletion alleviates electrophysiological dysfunction in 5XFAD mice (mean ± s.e.m.; n=6 in each group; *P<0.05). (d,e) ELISA quantification of Aβ in total brain lysates (d) or SDS fraction (e) from 6-month-old mice (mean ± s.e.m.; n=9 in 5XFAD, n=10 in 5XFAD/AEP−/− mice; *P<0.01). (f) Thioflavin-S staining showing the Aβ plaques in the hippocampus (HC) and frontal cortex (FC). Scale bar, 50 μm. (g,h) Quantification of number and surface area of Aβ plaques (mean ± s.e.m.; n=6; *P<0.01). (i) Morris water maze analysis as distance travelled (millimetres) and integrated distance (area under the curve, AUC) for WT (n=8), AEP−/− (n=8), 5XFAD (n=9) and 5XFAD/AEP−/− (n=10) mice (mean ± s.e.m.; *P<0.01). (j) Probe trial result. Shown is the mean ± s.e.m. percentage of time spent in the target quadrant (*P<0.05). Data were analysed using t-test (d,e,g,h) or one-way analysis of variance followed by post hoc comparison (a,b,c,i). TBS, theta-burst stimulation.
Deletion of AEP ameliorates memory deficits in 5XFAD mice.

To ascertain whether AEP influences the processing of APP and accumulation of Aβ peptides in vivo, we analysed total Aβ, Aβ40 and Aβ42 in the brains of 5XFAD/AEP−/− mice. We found ~30% reduction in all Aβ peptide species in 5XFAD/AEP−/− mice compared with 5XFAD mice at 6 months of age. In addition, the highly aggregated, SDS-extractable forms of Aβ peptides were also decreased in 5XFAD/AEP−/− mice (Fig. 7d,e).

Remarkably, the cerebral Aβ level was lower in 5XFAD/AEP−/− mice than in 5XFAD mice brain even at 1.5 months of age, before plaque deposition (Supplementary Fig. 10c). Thioflavin-S staining and Aβ immunohistochemistry both revealed fewer Aβ plaques in hippocampus and frontal cortex in 5XFAD/AEP−/− versus 5XFAD mice. Quantification of the Aβ plaque number and surface area demonstrated substantially reduced plaque burden in 5XFAD/AEP−/− mouse brain (Fig. 7g and Supplementary Fig. 10d,e). Immunoblotting analysis revealed that the AEP-derived APP fragments in 5XFAD mice were diminished in 5XFAD/AEP−/− mice brain. APP and BACE1 expression levels were similar between 5XFAD and 5XFAD/AEP−/− mice, but the C-terminal APP fragment (C99) resulting from BACE1 cleavage was decreased in 5XFAD/AEP−/− mice. These results indicate that AEP deletion reduces Aβ production by attenuating BACE1-mediated cleavage of APP (Supplementary Fig. 9b).

Next, we evaluated spatial memory abilities in 6-month-old WT, AEP−/−, 5XFAD and 5XFAD/AEP−/− mice using the Morris water maze test. As expected, 5XFAD mice showed longer latency periods and longer swim path distance than WT mice, representing deficits in spatial memory formation. However, 5XFAD/AEP−/− mice were largely protected from spatial memory impairment (Fig. 7i and Supplementary Fig. 10f). All the mice exhibited comparable swim speeds, indicating that AEP gene knockout does not affect motor function (Supplementary Fig. 10g). 5XFAD/AEP−/− mice also performed better than 5XFAD mice on the probe test, spending more time in the target quadrant and indicating better memory recall (Fig. 7j). To verify that the behavioural effects of 5XFAD/AEP−/− were not strain dependent, we also examined the rescue effect of AEP gene knockout in another well-established AD mouse model expressing two AD mutant genes, APP and PS1. APP/PS1/AEP−/− mice at 15 months of age showed shorter latency periods and swim path distance than APP/PS1 mice during the training sessions, and spent more time in the target quadrant during the probe test (Supplementary Fig. 11a–d). Thus, AEP gene knockouts protect against memory deficits in two different AD mouse models.

Preserved memory in mice expressing uncleavable APP. To confirm the role of AEP-mediated APP cleavage in the pathogenesis of AD, we injected WT mice with adeno-associated virus (AAVs) expressing human mutant APPSLA, encoding the Swedish, London and Austrian mutations that are associated with early-onset familial AD40 or an AEP-uncleavable form APPSLA/N373A/N585A. The AAVs were injected into the hippocampus, where they expressed the embedded cDNA specifically in pyramidal neurons under control of the human synapsin-1 promoter. The expression levels of APPSLA and APPSLA/N373A/N585A were similar 6 months after the intracerebral injection of the AAVs as indicated by immunohistochemistry and western blot using an antibody specific to human APP (Fig. 8a,b). In the Morris water maze probe test, the APPSLA/N373A/N585A mice spent more time in the target quadrant than did the APPSLA mice, indicating preserved cognitive function (Fig. 8c). Expression of APPSLA caused significant synaptic loss in the hippocampus, while the AEP-uncleavable N373A/N585A mutations ameliorated the synaptic loss (Fig. 8d). Electrophysiological analysis found that the paired pulse ratio was preserved in APPSLA/N373A/N585A mice as compared with the APPSLA mice (Fig. 8e). The magnitude of LTP was also significantly elevated in mice expressing uncleavable APPSLA/N373A/N585A (Fig. 8f). Furthermore, the slope of I/O curve was preserved in the APPSLA/N373A/N585A mice (Fig. 8g). Collectively, these results strongly support that the AEP-mediated processing of APP plays critical roles in the pathogenesis of AD.

Discussion

In the present study, we have identified AEP as a novel pro-amyloidogenic protease cleaving the APP ectodomain at both N373 and N585 sites. AEP cleavage of APP at N585 enhances subsequent BACE1 processing of the APP-generated APP stub (APP566–605), increasing Aβ levels. The APP-generated N-terminal ectodomain of APP1–373 triggers neurodegeneration, indicating that AEP activation may induce neuronal cell death through proteolytic processing of APP. Blockade of AEP processing of APP in a variety of paradigms protects against Aβ accumulation, synaptic loss and behavioural deficits. Since AEP expression and activation is elevated in brain in an age-dependent manner, and leads to increased cleavage of APP in aged brains (Fig. 6), we propose that AEP contributes to the strong effect of ageing on AD risk. Collectively, these observations provide a rationale for the development of AEP inhibitors for treating AD.

The accumulation of Aβ in AD brain is caused by an imbalance between its production and clearance41. There is direct evidence that Aβ clearance is impaired in late-onset sporadic AD42. On the other hand, there is also evidence demonstrating increased production of Aβ in familial Alzheimer’s disease caused by Swedish mutation of APP43. Late-onset sporadic AD cases show elevated BACE1 levels, which is the rate-limiting enzyme for Aβ production. The elevation of BACE1 activity is correlated with brain Aβ loads44,45. Since human clinical trials of improving Aβ clearance for AD have failed46,47, Aβ clearance defects might not be the major or sole contributor to AD onset and progression. Our study supports a pathogenic model in which AEP levels and activity increase with age, creating a novel cleavage of APP that increases BACE1 processing and Aβ production under physiological conditions in neurons in vitro and in vivo.

APP is processed by α-, β- and γ-secretases, generating amyloidogenic and non-amyloidogenic fragments. Secretase-independent processing pathways may also exist, in that the half-life of APP is very short and since not all APP is secreted48. APP is also a substrate of caspases49, but the impact of this processing on Aβ generation and/or AD pathology is still not well established50. APP is degraded in the lysosomes51,52, presumably mediated at least in part by lysosomal enzymes such as AEP or cathepsins. Interestingly, cathepsin B cleaves APP at β-secretase cleavage siteS31,53,54. Here we provide both molecular and biochemical evidence showing that AEP may act as a novel δ-secretase that mediates the proteolytic processing of APP.

Where does AEP processing APP occur? It has been shown previously that lysosomal enzymes are abnormally distributed in human AD patient brains53. AEP usually resides in endolysosomes in healthy cells, but it is secreted extracellularly or may be leaked into the cytoplasm in cancer cells, aged cells and in AD neurons21,26. We found that AEP co-fractionates with BACE1 and APP along with endolysosomal markers and that it binds APP in mouse brain (Fig. 4). Fitting with these observations, Aβ treatment also elicits AEP co-localization with APP in primary neurons (Fig. 4c). AEP is secreted extracellularly, and associates with APP on the cell surface. Cell surface-associated AEP can be internalized and processed to its active form (Supplementary Fig. 7).
Figure 8 | Blockade of AEP cleavage prevents synaptic and cognitive dysfunction induced by APP SLA. (a,b) AAV-mediated expression of human APP SLA and APP SLA N373AN585A detected by immunohistochemistry and western blot (WB) using an antibody specific to human APP. Scale bar, 100 µm. (c) Probe trial of Morris water maze test showing blockade of AEP cleavage prevents memory deficits induced by APP SLA (mean ± s.e.m.; *P < 0.05). (d) The synaptic density in the hippocampus of mice injected with AAV-APP SLA and AAV-APP SLA N373AN585A determined by electron microscopy (mean ± s.e.m.; n = 6; *P < 0.05, **P < 0.01). Scale bar, 1 µm. (e–j) Blockade of AEP cleavage preserves the electrophysiological function. The APP SLA N373AN585A mice showed higher ratio of paired pulse (e), higher averaged magnitude of LTP (f) and higher slope of I/O curve (g) than the APP SLA N373AN585A mice. (h) Possible pathways for AEP-mediated cleavage of APP, tau and SET in AD. (1) AEP might translocate from the endolysosome into the cytoplasmic space, where it cleaves tau; (2) intracellular AEP cuts SET, leading to PP2A inhibition and consequent tau hyperphosphorylation; (3) intracellular AEP can be fused with the endocytosed APP; (4) activated AEP might be secreted extracellularly, where it interacts with the ectodomain of APP; (5) AEP and APP can also form a complex, which can be internalized via endocytosis. Data were analysed using t-test (e,f,g) or one-way analysis of variance followed by post hoc comparison (d).
Given that APP cleavage by AEP promotes the subsequent BACE1 processing of APPβ66-695 via release of steric hindrance and more access to the substrate, AEP might act as an upstream/initializing trigger for APP amyloidogenic processing. Since Aβ processing is mainly mediated by BACE1 and γ-secretase, deletion of the upstream δ-secretase predominantly impairs APP cleavage efficiency by BACE1. In addition to APP, AEP also cleaves other substrates implicated in AD including tau and SET (IP22)9,21,23. The reduction of Aβ levels in 5XFAD/AEP−/− mice rescues spine density, LTP and cognitive function (Fig. 7).

On the basis of these findings, we propose the following model. During ageing and other cellular stressors in the AD brain, AEP translocates from the endolysosome into the cytoplasmic space, where it cleaves tau, resulting in truncated neurotoxic fragments, hyperphosphorylation and neurofibrillary tangle formation22. Moreover, AEP cleaves SET, leading to PP2A inhibition and accelerates tau hyperphosphorylation21. AEP is also secreted extracellularly and distributes in the CSF (Supplementary Fig. 7a). During the ageing process and in AD, the reduction in brain pH decreases and reduced cystein C levels both promote AEP proteolytic activation. The secreted AEP binds APP on the plasma membrane and cleaves APP at N373 and N585 residues, shedding a neurotoxic APPβ37-37 fragment. The AEP/APP complex may also be internalized via endocytosis, with the AEP further activated in the low-pH environment of the endosome, cleaving APP at the N585 site. The resultant APPβ66-695 fragment subsequently serves as an optimal substrate for BACE1, augmenting amyloid formation (Fig. 8h).

Consequently, the activated AEP and reduced inhibitory cystatin C levels in AD brains coordinately result in amyloid deposition and aggregation.

AEP expression and activity have been linked to a number of pathological conditions including atherosclerosis, cancer, stroke and neurodegenerative diseases19,21,56–58. Despite large-scale processing is mainly mediated by BACE1 and γ-secretase activity by AEP plays a crucial role in APP metabolism and in AD pathogenesis. Consequently, the activated AEP and reduced inhibitory cystatin C levels in AD brains coordinately result in amyloid deposition and aggregation.

AEP expression and activity have been linked to a number of pathological conditions including atherosclerosis, cancer, stroke and neurodegenerative diseases19,21,56–58. Despite large-scale efforts to therapeutically target putative disease mechanisms in AD, such as Aβ production or clearance, neuroprotective treatments are still lacking. There is growing consensus that gaining a better understanding of the underlying disease mechanisms is urgently needed. Our findings provide substantial in vitro and in vivo evidence demonstrating that blockade of AEP cleavage of APP reduces Aβ production and amyloid deposition, underscoring that δ-secretase activity by AEP plays a crucial role in APP metabolism and in AD pathogenesis. Since δ-secretase cleaves both APP and tau in an age-dependent manner and mediates the amyloid plaque and NFT pathology onset23, our findings highlight a previously unappreciated role of this novel secretase in AD progression. Because this enzyme also cleaves TDP-43 (ref. 22), this δ-secretase might also contribute to other age-dependent neurodegenerative diseases including some forms of frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Hence, inhibition of AEP may be a novel therapeutic strategy for treating several pathogenic mechanisms that contribute to AD and other neurodegenerative diseases.

Methods

**Transgenic mice.** 5XFAD mice and APP/PS1 mice on a C57BL/6) background were obtained from the Jackson Laboratory (Stock No. 006554 and 004462, respectively). AEP knockout mice on a mixed C57BL/6 and 129/Olaa background were generated as reported24. Only male mice were used. All mice were housed under standard conditions at 22 °C and a 12-h light/dark cycle with free access to food and water. Animal care and handling was performed according to the Declaration of Helsinki and the Emory Medical School Guidelines. The following animal groups were analysed: WT, AEP−/−, 5XFAD, 5XFAD/AEP−/−, APP/PS1 and APP/PS1/AEP−/−. Sample size was determined by Power and Precision (Biosoft). 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 eight AD cases (age 74.5 ± 11.2 years, mean ± s.d.) and eight non-demented controls (age 73.2 ± 12.7 years) from the Emory Alzheimer’s Disease Research Center. Informed consent was obtained from all subjects. The study was approved by the Emory University CND Tissue 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. The post-mortem interval was similar between the AD group and control group.

**Antibodies and reagents.** Antibodies to the following targets were used: anti-AEP N-terminal antibody (clone 22C11, 1:1,000, Calbiochem), myc (1:1,000, Calbiochem), anti-APP C-terminal antibody (1:500, CT15, from Dr Edward Koo, University of California59), GFP (1:1,000, Santa Cruz), and LAMP1 (1:500, Santa Cruz), GST-horseradish peroxidase (HRP), β-tubulin III, tubulin, MAP2 and Aβ (all 1:1,000, Sigma, Aldrich), presenilin-1 (1:500, Abcam), BACE1 (1:1,000, Cell Signaling), GGA3 (1:500, Cell Signaling), EEA1 (1:500, Cell Signaling), AEP antibody clone 6E3 (1:1,000, from Dr Colin Watts, University of Dundee17), human AEP antibody (1:200, R&D) and sAPPα (1:1,000, MBL). Inhibitors were used against: caspase (20 mM ZVAD-fmk, Calbiochem), γ-secretase (10 mM TAPI-1, Calbiochem), β-secretase (50 nM KETTESEVN(stat)VAEF, Calbiochem), γ-secretase (25 μM DAPT, Calbiochem) and cathepsin (10 μM E64, Sigma-Aldrich). Histostain-SP, mouse and human Aβ40 and Aβ42 ELISA kits were purchased from Invitrogen. Total Aβ chemiluminescent ELISA kit was purchased from ThermoFisher. The In Situ Cell Death Detection Kit was purchased from Roche. Recombinant AEP was purchased from Novoprotein. The recombinant AEP was first activated by incubation in activation buffer (0.1 M NaOAc, 0.1 M NaCl, pH 4.5) at 37 °C for 4 h. Recombinant BACE1 was purchased from Sigma-Aldrich. Recombinant ADAM10 was purchased from R&D, Z-Ala-Ala-Asn-AMC was purchased from Bachem. All chemicals not included above were purchased from Sigma-Aldrich.

**In vitro AEP cleavage assay.** To assess the cleavage of APP by AEP in vitro, HEK293 cells (obtained from the American Type Culture Collection (ATCC)) were transfected with 10 μg GFP-APP or GST-APP plasmids by the calcium phosphate precipitation method. Forty-eight hours after transfection, the cells were collected, washed once in PBS, lysed in lyss buffer (50 mM sodium citrate, 5 mM dithiothreitol (DTT), 0.1% CHAPS and 0.5% Triton X-100), and centrifuged for 10 min at 14,000 g at 4 °C. The supernatant was then incubated with mouse kidney lysates at pH 7.4 or 6.0 at 37 °C for 30 min. To measure the cleavage of purified APP fragments by AEP, BACE1 or ADAM10, GST-tagged APP full length or fragments were purified with glutathione beads. The purified APP was incubated with recombinant AEP (5 μg ml−1) in AEP buffer (50 mM citrate, 5 mM DTT, 0.1% CHAPS and 0.5% Triton X-100, pH 6.0), recombinant BACE1 (100 μl−1) in BACE1 buffer (20 mM sodium acetate, 0.1% Triton X-100, pH 4.5), or recombinant ADAM10 (1 ng ml−1) in ADAM10 buffer (25 mM Tris, 2 μM ZnCl2, 0.005% Brij 35, pH 9.0) for 30 min. The samples were then boiled in 1× SDS loading buffer and analysed by immunoblotting.

**AEP activity assay.** Tissue homogenates or cell lysates (10 μg) were incubated in 200 μl assay buffer (20 mM citric acid, 60 mM Na2HPO4, 1 mM EDTA, 0.1% CHAPS and 1 mM DTT, pH 6.0) containing 20 μg 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. The activity of AEP was expressed as the reading at 1 h minus the first reading.

**Western blot analysis.** The 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 Na2VO4, 50 mM NaF, 10 mM sodium pyrophosphate and 10 mM sodium β-glycerophosphate, supplemented with protease inhibitors cocktail), and centrifuged for 15 min at 16,000g. The supernatant was boiled in SDS loading buffer. After SDS-PAGE, the samples were transferred to a nitrocellulose membrane. Western blot analysis was performed with a variety of antibodies. Images have been cropped for presentation. Full-size images are presented in Supplementary Fig. 12.

**Co-immunoprecipitation.** The mouse brain tissue samples were lysed in lysis buffer and centrifuged for 15 min at 16,000g. The supernatant was incubated with anti-AEP antibody and protein A/G-agarose overnight at 4 °C. After extensive washing, the bound proteins were eluted from the beads by boiling in Laemmli sample buffer and subjected to western blot analyses.

**Subcellular fractionation.** Subcellular fractionation of mouse brain tissues was performed as described previously60. Briefly, 150 mg frontal cortical tissue was minced with a scalpel blade in 1 ml of homogenization buffer (0.25 M sucrose, 1 mM MgCl2, 10 mM Tris-HCl, pH 7.4, supplemented with protease inhibitor
cocktail). The buffer was discarded after centrifugation at 100g for 2 min. The tissues were suspended in 1.5 ml homogenization buffer and homogenized by successive passages through needles of increasing gauge number (19–26). The suspension was centrifuged at 1,000g for 10 min to discard nuclei and cellular debris. The supernatant was adjusted to 1.4 M sucrose, and incorporated into a discontinuous sucrose gradient consisting of the four following layers: 2 ml 2 M sucrose, 2.25 ml 1.4 M sucrose, 3.75 ml 1.2 M sucrose and 5 ml 0.8 M sucrose. The gradient was incubated for 2.5 h at 100,000g. Fourteen fractions were collected from the top of the gradient and stored as aliquots at −80 °C. Equal volumes of each fraction were boiled in 1× SDS loading buffer and analysed by immunoblotting.

**Mass spectrometry analysis.** Protein samples were in-gel digested with trypsin. Peptide samples were resuspended in loading buffer (0.1% formic acid, 0.05% trifluoroacetic acid and 1% acetonitrile) and injected onto a 20-cm high-performance liquid chromatography column (internal diameter 100 μm) packed with ReProfil-Pur 120 C18-AQ 1.9 μm beads (Dr. Maish). ESI and PEA were carried out at a 2 h 4–80% buffer B reverse phase gradient (buffer A: 0.1% formic acid and 1% acetonitrile in water; buffer B: 0.1% formic acid in acetonitrile) generated by a NanoAcuity UPLC system (Waters Corporation). 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) using a 10 precursor ions with charge determined by the acquisition software to be ≥2. Dynamic exclusion of peaks already sequenced was for 20 s with early expiration for two count events with signal to noise > 2. Automatic gating control was set to 150 ms maximum injection time or 106 counts. To identify AEP cleavage sites on human APP, the SageN SequEST 3.5 algorithm was used to search and match MS/MS spectra to a complete semi-tryptic human proteome database (NCBI reference sequence revision 50, with 66,652 entries) plus pseudo-reversed sequences61,62 with a FDR of 0.25% for proteins identified by more than 5 peptides, or ≥1% for a single peptide. The FDR was estimated by the number of decoy matches (nd) and total number of assigned matches (nt). FDR = 2 × nd/nt, assuming mismatches in the decoy database were the same as in the decoy database. To detect the AEP-derived APP fragments, AD brain lysates were immunoprecipitated with anti-APP N-terminal antibody and subjected to proteomic analysis. All semi-tryptic MS/MS spectra for putative AEP-generated APP cleavage sites were manually inspected.

**Immunohistochemistry.** Free-floating 30-μm-thick serial sections were treated with 0.3% hydrogen peroxide for 10 min. Then, sections were washed three times in PBS. Sections were blocked in 1% BSA and 0.3% Triton X-100, for 30 min followed by incubation in 1% sodium hydroxide and 0.9% hydrogen peroxide for 20 min. After rinsed in distilled water, the sections were transferred to blocking solution containing 1% bovine serum albumin and 0.03% TBS for 1 min and removed; the coated plates were blocked by adding 1% BSA solution in PBS (0.1 M Tris- HCl, pH 7.5, 150 μM MgCl2, 1% NP-40, 0.5% NaDOC and 0.1% SDS), centrifuged at 100,000 rpm for 30 min and the pellet containing insoluble Aβ was further extracted in 2% SDS, 25 mM Tris-HCl, pH 7.5.

**ELISA quantification of Aβ.** To detect the concentration of Aβ in total brain lysates, the mouse brains were homogenized in 8× mass of 5 M guanidine HCl/50 mM Tris-HCl (pH 8.0), and incubated at room temperature for 3 h. Then the homogenate was diluted with cold reaction buffer (PBS with 5% BSA and 0.03% Tween-20, supplemented with protease inhibitor cocktail) and centrifuged at 16,000g for 20 min at 4 °C. The Aβ in the total brain and the Aβ in the SDS fraction were analysed with human Aβ42 (KHB3441, Invitrogen), Aβ40 (KHB3481, Invitrogen) and total Aβ (SIG-38966, Covance) ELISA kits according to the manufacturer’s instructions. The Aβ concentrations were determined by comparison with the standard curve. To assess the effect of AEP on Aβ production, HEK293 cells stably transfected with human APP695 were transfected with small interfering RNA (siRNA) against AEP using lipofection 2000 reagent. AEP 27mer siRNA was purchased from Origene. The siRNA sequences are rCrGrArGrAr UrGrCmUrPrStGrArTrGTT, ArGrCrGrArGrUrCrArGrArTrGCG, ArGrCrGrArGrUrCrArGrArTrGCG. Non-targeting control (rCrGrUrAr UrGrCrGrCrGrArUrArUrArUrCrGrGrArTrArT) was transfected in parallel as control. To detect the Aβ production by different APP fragments, HEK293 cells were transfected with GST-APP full length, GST-APP-K40, GST-APP-N40, GEP-APP, GEP-APP-N337A and GEP-APP N858A, respectively. Twenty-four hours post-transfection, the cells were fed with fresh media. Media were collected after conditioning for 24 h, and cell debris was removed by centrifugation. Complete protease inhibitor cocktail (Roche) was added and Aβ40 and Aβ42 were quantified with human Aβ42 and Aβ40 ELISA kits (KHB3441 and KHB3481, Invitrogen). The Aβ concentrations of Aβ42 and Aβ40 in AEP+/− and AEP−/− mouse neocortical were measured using mouse Aβ42 and Aβ40 ELISA kits (KMB3441 and KMB3481, Invitrogen).

**ELISA quantification of APP fragments.** The ELISA was carried out by using 96-well Nunc-Immuno MaxiSorp plates for TMB (3,3′,5,5′-tetramethylbenzidine from Sigma Catalogue T0525). These plates were coated with 100 μl antibody that specifically recognize AEP-derived APP fragments between 1-2000. The plates were incubated overnight at 4 °C and washed three times with PBS with 0.05% Tween-20 (PBST) for 1 min each. The plates were blocked by adding 200 μl well of 2% BSA in PBST for 2 h at room temperature. After blocking, 100 μl well of samples diluted in 2% BSA/PBST (+ 2 mM Na3VO4) were added to each well and the plate was incubated overnight at 4 °C and washed four times with PBST. After washing, the plates were incubated overnight at 4 °C with a 1:1000 dilution of 1× 2% BSA/PBST and the plates were incubated for 2 h at room temperature. After blocking, 100 μl well of samples diluted in 2% BSA/PBST (+ 2 mM Na3VO4) were added to each well and the plate was incubated overnight at 4 °C and washed four times with PBST. After washing, the plates were incubated overnight at 4 °C with a 1:1000 dilution of 1× 2% BSA/PBST and the plates were incubated for 2 h at room temperature. After blocking, 100 μl well of samples diluted in 2% BSA/PBST (+ 2 mM Na3VO4) were added to each well and the plate was incubated overnight at 4 °C and washed four times with PBST. After washing, the plates were incubated overnight at 4 °C with a 1:1000 dilution of 1× 2% BSA/PBST and the plates were incubated for 2 h at room temperature. After blocking, 100 μl well of samples diluted in 2% BSA/PBST (+ 2 mM Na3VO4) were added to each well and the plate was incubated overnight at 4 °C and washed four times with PBST. After washing, the plates were incubated overnight at 4 °C with a 1:1000 dilution of 1× 2% BSA/PBST and the plates were incubated for 2 h at room temperature. After blocking, 100 μl well of samples diluted in 2% BSA/PBST (+ 2 mM Na3VO4) were added to each well and the plate was incubated overnight at 4 °C and washed four times with PBST. After washing, the plates were incubated overnight at 4 °C with a 1:1000 dilution of 1× 2% BSA/PBST and the plates were incubated for 2 h at room temperature. After blocking, 100 μl well of samples diluted in 2% BSA/PBST (+ 2 mM Na3VO4) were added to each well and the plate was incubated overnight at 4 °C and washed four times with PBST. After washing, the plates were incubated overnight at 4 °C with a 1:1000 dilution of 1× 2% BSA/PBST and the plates were incubated for 2 h at room temperature.
well of anti-mouse HRP-conjugated secondary antibody (Fisher) 1:500 diluted in PBS for 30 min. Following three washes in PBS, the slides were incubated with Texas Red-conjugated anti-mouse (Amersham Biosciences). The slides were washed three times in PBS, then the sections were mounted with Fluoromount-G (Southern Biotech) and observed using a Leica DMR fluorescence microscope or a Zeiss LSM 510confocal microscope.

**Primary neuron culture.** Primary neurons were dissected from E18 embryos and cultured as described previously. To measure the effect of APP fragments on neuronal survival, primary cortical neurons cultured in vitro (DIV 12) were exposed to different concentrations of APP fragments for 24 h. The neurons were then fixed in 4% formaldehyde, permeabilized and immunostained with anti-MAP2 antibody (1:1,000). Pictures of the neurons were taken by fluorescence microscopy. Neuronal survival index was expressed as the percentage of TUNEL-positive neurons out of the total number of MAP2-positive neurons.

**AAV vector packaging.** AAV vectors carrying human APP SLA were gifts from Dr Kuegler from Max Planck Institute of Psychiatry, Germany.40 The vectors were used in Azimuth cDNA (Fisher Scientific). The vectors were amplified by double-stranded DNA in 100 ml of TMB solution to dissolve TMB 1 mg tablet in 1 ml dimethyl sulfoxide, and freshly diluted to 9 ml P 5.0 citrate buffer (to 50 ml de-ionized water, add 20 ml of 0.1 M citric acid plus 29.5 ml 0.1 M sodium citrate, mix well) and add 100 ml of 3% H2O2 was added to each well and incubated at 37 C for 10–30 min. The reaction was stopped by adding 50 ml of 3N HCl and then the slides of each well were recorded using microplate reader at 450 nm or luminences, and subtract the readings at 650 nm from the readings at 450 nm. This subtraction will correct the optical imperfections in the plate.

**Immunofluorescence.** Cultured neurons were fixed in 4% formaldehyde for 10 min, washed with PBS, blocked in 1% BSA and 0.3% Triton X-100 for 30 min followed by overnight incubation with primary antibodies at 4 C. The slides were washed three times in PBS, then incubated for 1 hr with secondary antibody (1:5,000 diluted in PBS). The slides were washed three times in PBS, then the sections were covered with a glass cover using mounting solution and examined using a fluorescence microscope or confocal microscopy (Olympus).

**Statistical analysis.** All the quantitative data were presented as mean ± s.e.m. Statistical analysis was performed using either Student's t-test (two-group comparison) or one-way analysis of variance (more than two groups) followed by post hoc comparison, and differences with P values < 0.05 were considered significant.

**References**

1. Selkoe, D. J. Amyloid beta-protein and the genetics of Alzheimer's disease. J. Biol. Chem. 271, 18295–18298 (1996).
2. Huang, C. Take five—Beta-secretase: the gamma-secretase quartet conduct Alzheimer’s amyloid beta-peptide generation. EMBO J. 23, 483–488 (2004).
3. Kang, J. et al. The precursor of Alzheimer’s disease amyloid A4 protein resembles a cell-surface receptor. Nature 325, 733–736 (1987).
4. Dykxhoorn, D. M. Identification, transmembrane orientation and biogenesis of the amyloid A4 precursor of Alzheimer's disease. EMBO J. 7, 949–957 (1988).
5. Haass, C., Kastner, C., Thal, D. & Selkoe, D. J. Trafficking and proteolytic processing of APP. Cold Spring Harb. Perspect. Med. 2, a006270 (2012).
6. Vassar, R. RACE1: the beta-secretase enzyme in Alzheimer's disease. J. Mol. Neurosci. 23, 105–114 (2004).
7. Hong, Y. et al. Structure of the protease domain of memapsin 2 (beta-secretase) complexed with inhibitor. Science 290, 150–153 (2000).
8. Vassar, R. et al. Beta-secretase cleavage of Alzheimer’s amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286, 735–741 (1999).
9. Kaether, C., Haass, C. & Steiner, H. Assembly, trafficking and function of the secreted form of the amyloid beta protein precursor. J. Biol. Chem. 272, 27857–27864 (1997).
10. Weidemann, A. Novel cell-permeable acyloxymethylketone inhibitors of asparagine endopeptidase. J. Biol. Chem. 272, 38980–38990 (2003).
11. Kuhn, P. H. et al. ADM10 is the physiologically relevant, constitutive alpha-secretase of the amyloid precursor protein in primary neurons. EMBO J. 29, 3020–3032 (2010).
12. Lammich, S. et al. Constitutive and regulated alpha-secretase cleavage of the Alzheimer’s amyloid precursor protein by a disintegrin metalloproteinase. Proc. Natl Acad. Sci. USA 96, 3922–3927 (1999).
13. Shen, C. et al. Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase. J. Biol. Chem. 272, 8090–8097 (1997).
14. Pampaloni, F. & Kolodner, R. D. Identification and characterization of a yeast homologue of mammalian legumain. J. Biol. Chem. 271, 13377–13385 (1996).
15. Li, D. N., Matthews, S. P., Antoniou, A. N., Mazzeo, D. & Watts, C. Multistep autoactivation of asparaginyl endopeptidase in vitro and in vivo. J. Biol. Chem. 278, 38890–38899 (2003).
16. Shihraoma-Nada, K. et al. Biosynthetic processing of cathepsins and lysosomal degradation are abolished in asparaginyl endopeptidase-deficient mice. J. Biol. Chem. 278, 33194–33199 (2003).
17. Liu, Z. et al. Neuroprotective actions of PIKE-L by inhibition of SET proteolytic degradation by asparaginyl endopeptidase. Mol. Cell 29, 665–678 (2008).
18. Madeira, A., Pommel, J. M., Proehlant, A. & Allianquent, B. SET secretase (TAF1beta, I2PP2A) is involved in mouse apoptosis induced by an amyloid precursor protein cytoplasmic subdomain. FASEB J. 19, 1005–1007 (2005).
19. Basurot-Islas, G., Grundk-Isbql, I., Tung, Y. C., Liu, F. & Isbqal, K. Activation of asparaginyl endopeptidase leads to tau hyperphosphorylation in Alzheimer disease. J. Biol. Chem. 288, 17495–17507 (2013).
20. Herskowitz, J. H. et al. Asparaginyl endopeptidase cleaves TDP-43 in brain. Proteomics 12, 2455–2463 (2012).
21. Zhang, Z. et al. Cleavage of tau by asparagine endopeptidase mediates the neurofibrillary pathology in Alzheimer’s disease. Nat. Med. 20, 1254–1262 (2014).
22. Levy, E. & Alhmad, I. Cystatin C in Alzheimer’s disease. Biochimie 79 (2012).
23. Levy, E. et al. Codopoination of cystatin C with amyloid-beta protein in the brain of Alzheimer disease patients. J. Neuropathol. Exp. Neurol. 60, 94–104 (2001).
24. Levy, E. & Alhmad, I. Cystatin C in Alzheimer’s disease. Front. Mol. Neurosci. 5, 79 (2012).
25. Smith, R. et al. Intracerebrovascular regulation of activity and processing of legumain by cystatin E/M. Biochim. Biophys. Acta 150–153 (1999).
26. Zhao, L., et al. Structural analysis of asparaginyl endopeptidase reveals the activation mechanism and a reversible intermediate maturation stage. Cell Res. 24, 344–358 (2014).
27. Loak, K. et al. Novel cell-permeable acyloxymethylketone inhibitors of asparaginyl endopeptidase. Biol. Chem. 384, 1239–1243 (2009).
28. Zhang, Y. et al. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. J. Neurosci. 34, 11949–11967 (2014).
29. Weidemann, A. et al. Proteolytic processing of the Alzheimer’s disease amyloid precursor protein within its cytoplasmic domain by caspase-like proteases. J. Biol. Chem. 274, 5823–5829 (1999).
31. Mueller-Steiner, S. et al. Antiamyloidogenic and neuroprotective functions of cathepsin B: implications for Alzheimer’s disease. *Neuron* **51**, 703–714 (2006).
32. Tesco, G. et al. Depletion of GGA3 stabilizes RACE and enhances beta-secretase activity. *Neuron* **54**, 721–737 (2007).
33. Tu, S., Okamoto, S., Lipton, S. A. & Xu, H. Oligomeric Aβ induces synaptic dysfunction in Alzheimer’s disease. *Mol. Neurodegener.* **9**, 48 (2014).
34. Smith, R. et al. Intracellular localization of amyloid-β peptide in SH-SY5Y neuroblastoma cells. *J. Alzheimers Dis.* **37**, 713–733 (2012).
35. Nikolaev, A., McLaughlin, T., O’Leary, D. D. & Tessier-Lavigne, M. APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. *Nature* **457**, 981–989 (2009).
36. Haass, C. & Selkoe, D. J. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer’s amyloid beta-peptide. *Nat. Rev. Mol. Cell Biol.* **8**, 101–112 (2007).
37. Shankar, G. M. & Walsh, D. M. Alzheimer’s disease: synaptic dysfunction and Abeta. *Mol. Neurodegener.* **4**, 48 (2009).
38. Hongpaisan, J., Sun, M. K. & Alkon, D. L. PKC epsilon activation prevents synaptic loss, Abeta elevation, and cognitive deficits in Alzheimer’s disease transgenic mice. *J. Neurosci.* **31**, 630–643 (2011).
39. Kincaid, R. & Benson, M. Impairments in remote memory stabilization precede hippocampal synaptic and cognitive failures in 5XFAD Alzheimer mouse model. *Neurobiol. Dis.* **32**, 229–235 (2009).
40. Jaworski, T. et al. AAV-tau mediates pyramidal neurodegeneration by cell-cycle re-entry without neurofibrillary tangle formation in wild-type mouse. *PloS ONE* **4**, e7280 (2009).
41. Hardy, J. & Selkoe, D. J. The amyloid hypothesis of Alzheimer’s disease: progress and problems on the road to therapeutics. *Science* **297**, 353–356 (2002).
42. Mawuenyega, K. G. et al. Decreased clearance of CNS β-amyloid in Alzheimer’s disease. *Science* **330**, 1774–1774 (2010).
43. Nilbert, C. et al. The Arctic APP mutation (E693G) causes Alzheimer’s disease by enhanced Aβ protofibril formation. *Nat. Neurosci.* **4**, 887–893 (2001).
44. Yang, L.-B. et al. Elevated β-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. *Nat. Med.* **9**, 3–4 (2003).
45. Li, R. et al. Amyloid β peptide load is correlated with increased β-secretase activity in sporadic Alzheimer’s disease patients. *Proc. Natl Acad. Sci. USA* **101**, 3632–3637 (2004).
46. Silverberg, G. et al. Continuous CSF drainage in AD results of a double-blind, randomized, placebo-controlled study. *Neurology* **71**, 202–209 (2008).
47. Kurz, A. & Perneeczky, R. Amyloid clearance as a treatment target against Alzheimer’s disease. *J. Alzheimers Dis.* **24**, 61–73 (2010).
48. Weidemann, A. et al. Identification, biogenesis, and localization of precursors of Alzheimer’s disease A4 amyloid protein. *Cell* **57**, 115–126 (1989).
49. Lu, D. C., Soriano, S., Bredesen, D. E. & Koo, E. H. Caspase cleavage of the amyloid precursor protein to potentially amyloidogenic derivatives. *J. Neurochem.* **87**, 753–741 (2003).
50. Harris, J. A. et al. Many neuronal and behavioral impairments in transgenic mouse models of Alzheimer’s disease are independent of caspase cleavage of the amyloid precursor protein. *J. Neurosci.* **30**, 372–381 (2010).
51. Golde, T. E., Estus, S., Younkin, L. H., Selkoe, D. J. & Younkin, S. G. Processing of the amyloid protein precursor to potentially amyloidogenic derivatives. *Science* **255**, 728–730 (1992).
52. Haass, C., Koo, E. H., Mellon, A., Hung, A. Y. & Selkoe, D. J. Targeting of cell-surface beta-amyloid precursor protein to lysosomes: alternative processing into amyloid-bearing fragments. *Nature* **357**, 500–503 (1992).
53. Hook, V. Y., Kindy, M. & Hook, G. Inhibitors of cathepsin B improve memory and reduce beta-amyloid in transgenic Alzheimer disease mice expressing the wild-type, but not the Swedish mutant, beta-secretase site of the amyloid precursor protein. *J. Biol. Chem.* **283**, 7745–7753 (2008).
54. Hook, V. Y., Kindy, M., Reineckel, T., Peters, C. & Hook, G. Genetic cathepsin B deficiency reduces beta-amyloid in transgenic mice expressing human wild-type amyloid precursor protein. *Biochem. Biophys. Res. Commun.* **386**, 284–288 (2009).
55. Cataldo, A. M., Paskevich, P. A., Kominami, E. & Nixon, R. A. Lysosomal hydrolases of different classes are abnormally distributed in brains of patients with Alzheimer disease. *Proc. Natl Acad. Sci. USA* **88**, 10998–11002 (1991).
56. Clérin, Y. et al. Expression of the cysteine protease legumain in vascular lesions and functional implications in atherosclerosis. *Atherosclerosis* **201**, 53–66 (2008).
57. Holland, M. et al. RAC2, AEP, and ICAM1 expression are associated with CNS disease in a mouse model of pre-B childhood acute lymphoblastic leukemia. *Blood* **118**, 638–649 (2011).
58. Chan, C. B. et al. Mice lacking asparaginyl endopeptidase develop disorders res.e.m.blig hemophagocytic syndrome. *Proc. Natl Acad. Sci. USA* **106**, 468–473 (2009).
59. Sisodia, S., Koo, E., Hoffman, P., Perry, G. & Price, D. Identification and transport of full-length amyloid precursor proteins in rat peripheral nervous system. *J. Neurosci.* **13**, 3136–3142 (1993).
60. Santosa, C. et al. Decreased expression of GGA3 protein in Alzheimer’s disease frontal cortex and increased co-distribution of RACE with the amyloid precursor protein. *Neurobiol. Dis.* **43**, 176–183 (2011).
61. Elias, J. E. & Gygì, S. P. Target-decoy strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat. Methods* **4**, 207–214 (2007).
62. Xu, P., Daoung, D. M. & Peng, J. Systematical optimization of reverse-phase chromatography for shotgun proteomics. *J. Proteome Res.* **8**, 3944–3950 (2009).
63. Zhang, Z. et al. 7,8-Dihydroxyflavone prevents synaptic loss and memory deficits in a mouse model of Alzheimer’s disease. *Neuropsychopharmacology* **39**, 638–650 (2014).