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ORIGINAL ARTICLE

p75NTR ectodomain is a physiological neuroprotective molecule against amyloid-beta toxicity in the brain of Alzheimer's disease

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In Alzheimer's disease (AD), neurodegenerative signals such as amyloid-beta (Aβ) and the precursors of neurotrophins, outbalance neurotrophic signals, causing synaptic dysfunction and neurodegeneration. The neurotrophin receptor p75 (p75NTR) is a receptor of Aβ and mediates Aβ-induced neurodegenerative signals. The shedding of its ectodomain from the cell surface is physiologically regulated; however, the function of the diffusible p75NTR ectodomain (p75ECD) after shedding remains largely not known. Here, we show that p75ECD levels in cerebrospinal fluid and in the brains of Alzheimer's patients and amyloid-beta precursor protein (APP)/PS1 transgenic mice were significantly reduced, due to inhibition of the sheddase-tumor necrosis factor-alpha-converting enzyme by Aβ. Restoration of p75ECD to the normal level by brain delivery of the gene encoding human p75ECD before or after Aβ deposition in the brain of APP/PS1 mice reversed the behavioral deficits and AD-type pathologies, such as Aβ deposit, apoptotic events, neuroinflammation, Tau phosphorylation and loss of dendritic spine, neuronal structures and synaptic proteins. Furthermore, p75ECD can also reduce amyloidogenesis by suppressing β-secretase expression and activities. Our data demonstrate that p75ECD is a physiologically neuroprotective molecule against Aβ toxicity and would be a novel therapeutic target and biomarker for AD.

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INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia and at present there is no disease-modifying therapy.[1,2] Amyloid-beta peptide (Aβ) has a central role in the pathogenesis of AD; however, the regulation of its production and clearance is not fully understood.[3] The dysregulation of neurotrophins and their receptors in AD is a key pathological process in the development of sporadic AD,[4–7] where neurotrophic signals consisting of mature neurotrophins and Trk receptors are downregulated.[6,8–10] Conversely, neurodegenerative signals such as Aβ, the precursor of nerve growth factor (proNGF) and their receptors, neurotrophin receptor p75 (p75NTR) and sortilin, are increased.[11–14] p75NTR is the neurotrophin receptor responsible for mediating the survival or apoptosis of neurons depending on relative expression levels of high affinity neurotrophin Trk receptors.[15,16,17,18] p75NTR signaling has critical roles in the plasticity of nerve innervation during development[19] and neurodegeneration after a nerve injury.[8,20] p75NTR is also a co-receptor of sortilin for proneurotrophins mediating apoptosis,[21] and of the Nogo receptor for Nogo-66 and myelin associated glycoprotein mediating neurite collapse.[22] p75NTR also binds with Aβ and regulates Aβ-induced degeneration of cholinergic neurons.[23–25] The ectodomain (p75ECD) shedding of p75NTR is physiologically regulated by tumor necrosis factor-alpha-converting enzyme (TACE), followed by regulated-intramembranous proteolysis by gamma-secretase.[26–28] However, the physiological and pathological significance of p75NTR shedding is not known. The regulation of p75NTR shedding and the function of diffusible p75ECD after shedding *in vivo* are yet to be determined. Here, we demonstrate that p75ECD is a neuroprotective factor, and levels of p75ECD are reduced in the brains of AD cases. The restoration of p75ECD levels alleviates AD pathologies and improves learning and memory in both early and later phases of AD in an APP/PS1 mouse model.

MATERIALS AND METHODS

The detailed materials and methods are presented in the Supplementary Information. Briefly, all human and animal studies were approved by respective ethic committees of Third Military Medical University and University of South Australia. Post-mortem human brain samples from histologically confirmed cases of AD and age-matched nondemented individuals were obtained from Banner Sun Health Research Institute (Sun City, AZ, USA). Cerebrospinal fluid (CSF) samples were obtained from

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patients diagnosis with AD and age-matched nondemented individuals in Daping Hospital. Adeno-associated virus (AAV)-p75ECD-Fc or AAV-EGFP viral particles were produced by Virovek (Hayward, CA, USA) (Supplementary Figure 1) and injected into the lateral ventricles of amyloid-beta precursor protein (APP)/PS1 transgenic AD mice at three (prevention) or nine (treatment) month old. All experiments including behavioral tests, tissue sampling, histological and biochemical analysis were performed by 12-months old as described.[29] Golgi stain was performed with a protocol described previously.[30] p75ECD was quantified by enzyme-linked immunosorbent assay or western blots. The levels of cytokines and Aβ in the brain and blood were quantified by enzyme-linked immunosorbent assay. Effects of p75NTR on APP processing and Tau phosphorylation were analyzed in cultured cortical neurons of p75NTR wild-type (Wt) and knockout AD mice. The effects of p75ECD-Fc on Aβ aggregation and disaggregation were examined using ThT and transmission electronic microscopy methods. Effect of p75ECD-Fc on neuronal toxicity, neurite growth, APP processing, β-site APP-cleaving enzyme (BACE1) expression, Tau phosphorylation and GSK3β activation in response to Aβ were examined in SH-SY5Y cells and mouse primary cortical neurons. Statistical comparisons between groups were assayed using Tukey's test, Students *t*-test, one-way analysis of variance, or two-way repeated-measures analysis of variance for testing the significance of values. *P*-values < 0.05 were considered significant. All these analyses were performed by using SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Shedding of p75ECD is downregulated in the brains of AD subjects and APP/PS1 mice

Soluble p75ECD was found to be present in many different regions of the brain (Supplementary Figures 2a and c) of Wt mice. The levels of p75ECD in the whole brain or different regions increased with age in Wt mice (Supplementary Figures 2a and b). The data indicates that p75NTR shedding is a physiological process and is regulated by ageing. The presence of p75ECD in the brain was confirmed in AD subjects and APP/PS1 (AD) mice (Figures 1a and d). p75ECD levels in the parietal cortex were significantly reduced ($P < 0.05$), whereas the full length (FL) p75NTR (p75FL) was significantly increased in AD patients ($P < 0.01$, $n = 12$) compared with the age- and gender-matched nondemented controls ($n = 12$, Figure 1b). Most importantly, the p75ECD levels in the CSF from AD patients ($n = 29$) were also significantly lower than the CSF from age-and gender-matched nondemented controls ($n = 27$, $P < 0.01$) (Figure 1a). The sensitivity of the enzyme-linked immunosorbent assay assay for p75ECD reached 150 pg/ml and it is specific as verified by western blots using the same samples of mouse brains ($r = 0.8198$, $P < 0.0001$) (Supplementary Figures 2d and e). The abnormal expression of p75ECD and p75FL in the brain of 12-month-old AD mice was remarkably similar to the AD brain in humans, that is, increase in p75FL but decrease in p75ECD compared with their Wt littermates (Figures 1c and d). The p75ECD:p75FL ratio was significantly reduced in both AD mice and patients (Figures 1b and d). These results indicate that the processing of p75NTR in AD was significantly reduced. As TACE is the main enzyme which cleaves the ECD of p75NTR,[28] we found that TACE activity was significantly reduced in the brain of AD mice and in CSF of AD patients (Figures 1e and g). TACE expression in AD mice was also reduced, compared with the age-matched Wt controls (Figure 1f). In contrast with NGF, which increases TACE-dependent cleavage of p75NTR,[27,31] Aβ treatment of mouse cortical neurons significantly reduced TACE expression in a dose-dependent manner (Figure 1h) and shedding of p75ECD (Figure 1i). Thus, a reduction in soluble p75ECD in AD is a downstream toxic action of Aβ.

Restoration of p75ECD levels in the brains of AD mice by delivery of the recombinant human ECD-Fc gene using AAV8 vector

Given our previous findings that recombinant p75ECD fused with human IgG Fc (ECD-Fc) can disrupt Aβ-p75NTR interaction and inhibit Aβ aggregation,[29] and that p75ECD levels were reduced in the AD brain as described above, we proposed that the restoration of p75ECD levels in AD brains may protect neurons against the pathology caused by Aβ and facilitate removal of Aβ deposits. AAV8 vector was used to deliver the fusion gene of human p75ECD and human IgG Fc fragment (AAV-ECD-Fc) into the left lateral ventricle of AD mice at 3 months (when no amyloid plaque is seen in the brain) or 9 months of age (when amyloid plaques are well developed and cognition is impaired), at dose of 2×10^9 vector genomes, which is equivalent to the dose (3×10^{11} viral genome per 60-kg human) used in human clincial trials of AAV.[32] AAV expressing the green fluorescent protein (GFP) gene (AAV-GFP) at the same dose was used as a control. To track the distribution of AAV, the same volume of Trypan blue was injected into the left lateral ventricle of mice and the injected dye diffused widely into both hemispheres and brain stem (Supplementary Figure 3). Expression of the ECD-Fc transgene was detected at one week after delivery, and remained stable in neurons of the neocortex, hippocampus and thalamus, up to 12 months of age when animals were culled (Supplementary Figures 4a, b, d and e). This was detected using an anti-human Fc antibody which only detects the ECD-Fc expressed from the transgene but not endogenous p75ECD (Supplementary Figures 4c and 5). The transgenes of ECD-Fc and GFP were widely expressed throughout different brain regions (Supplementary Figures 4a, d, 6). The levels of ECD-Fc in the brain of 12-month old AD mice after the transfection were similar to ECD levels of age-matched Wt mice (Supplementary Figure 4f).

Restoration of brain p75ECD levels prevents cognitive decline in AD mice

Compared with AAV-GFP-treated mice, the mice treated with AAV-ECD-Fc at 3 and 9 months of age, for prevention and treatment respectively, performed better in the Morris Water Maze test. This was reflected by a significant reduction in the escape latency time and the distance traveled to escape onto the platform with progressive platform learning trials, greater number of platform area crossings and more time spent in the platform quadrants in the probe trials without the platform (Figures 2a and b and Supplementary Figures 7 and 8). The data indicates that the AAV-ECD-Fc treatment significantly improves spatial learning and memory in AD mice. The mice in both prevention and treatment groups also performed better in Y-maze and open-field tests than the AAV-GFP-treated controls. This was reflected by more entries into the novel arm and higher spontaneous alternation in the Y-maze test (Figure 2c and Supplementary Figure 9). It indicates that the working memory of the AAV-ECD-Fc treated mice was better than that of the control mice. In the open-field test, a higher number of rearings was observed in the AAV-ECD-Fc treated mice, as well as a longer traveling distance and reduced time spent in the central zone, compared with the control mice (Figure 2d and Supplementary Figure 10), being similar to Wt mice. The data indicates that the ECD-Fc-treated mice have better general locomotor activity than the control AD mice.

Restoration of brain p75ECD levels reduces Aβ burdern in the brain and blood of AD mice

The amyloid plaque loads in the hippocampus and neocortex, identified by Congo red staining or immunohistochemistry, were reduced in AAV-ECD-Fc treated AD mice of prevention and treatment groups relative to the AAV-GFP treated controls, and were similar to plaque load of the 9-months-old untreated controls (Figures 3a and d). The profiles of cerebral amyloid angiopathy (CAA) and microhemorrhage were also reduced by ~ 50% in both prevention and treatment groups (Figures 3e and f). Interestingly, ECD-Fc was localized around and within Congo red or Thioflavine S positive plaques (Figure 3g), suggesting that

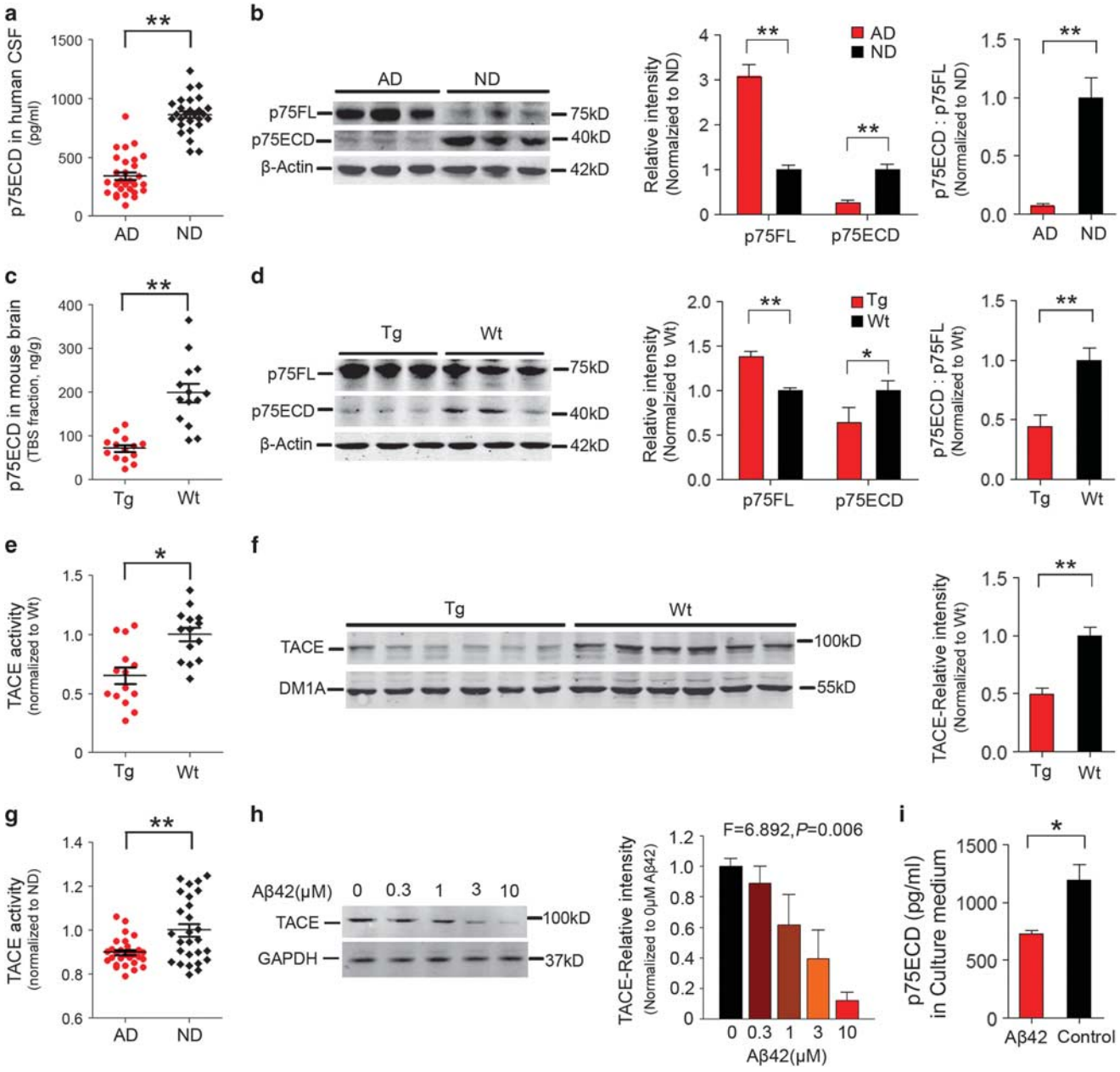


Figure 1. p75NTR ectodomain shedding in the brains of Alzheimer's disease patients and mice is downregulated by the inhibition of tumor necrosis factor-alpha-converting enzyme (TACE) activity induced by amyloid-beta. (**a**) Enzyme-linked immunosorbent assay of p75NTR ectodomain in cerebrospinal fluid of Alzheimer's disease patients and age-matched nondemented controls ($n = 29$ for Alzheimer's disease, $n = 27$ for nondemented, mean ± s.e.m., Student's *t*-test, $^{**}P < 0.01$). (**b**) Western blot of full length p75NTR in Alzheimer's disease and nondemented brain tissues, and the quantitative analyses of full length p75NTR, p75NTR ectodomain and p75NTR ectodomain:full length p75NTR ratio ($n = 12$ for Alzheimer's disease, $n = 12$ for nondemented, mean ± s.e.m., Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$). (**c**) Enzyme-linked immunosorbent assay analysis of p75NTR ectodomain in Tris-buffered saline fraction of brain homogenates of 12-month-old Alzheimer's disease mice (Tg) and wild-type littermates ($n = 14$ for Tg, $n = 14$ for wild type, mean ± s.e.m., Student's *t*-test, $^{**}P < 0.01$). (**d**) Western blot of p75NTR on brain tissues from 12-month-old Tg and wild-type mice, and the quantitative analysis of full length p75NTR, p75NTR ectodomain and p75NTR ectodomain:full length p75NTR ratio ($n = 13$ for Tg, $n = 14$ for wild type, mean ± s.e.m., Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$). (**e**) TACE activity of brain homogenates of 12-month-old Tg and wild-type mice assessed by fluorimetric assay ($n = 14$ for Tg, $n = 14$ for wild type, mean ± s.e.m., Student's *t*-test, $^{*}P < 0.05$). (**f**) Western blot of TACE in Tg and wild-type mice brain and quantitative analysis ($n = 14$ for Tg, $n = 14$ for wild type, mean ± s.e.m., Student's *t*-test, $^{**}P < 0.01$). (**g**) TACE activity in cerebrospinal fluid of Alzheimer's disease patients and nondemented controls ($n = 29$ for Alzheimer's disease, $n = 27$ for nondemented, mean ± s.e.m., Student's *t*-test, $^{**}P < 0.01$). (**h**) Western blot and quantification of TACE in extracts of cultured mouse primary cortical neurons treated with different doses of Aβ42 ($n = 3$, mean ± s.e.m., one-way analysis of variance, Tukey's test, $^{*}P < 0.05$). (**i**) Enzyme-linked immunosorbent assay analysis of p75NTR ectodomain in culture medium of mouse cortical neurons treated with or without 1.0-μm-Aβ42 ($n = 3$ per group, mean ± s.e.m., Student's *t*-test, $^{*}P < 0.05$).

expressed human ECD-Fc are associated with Aβ plaques. Consistent with the histological results, enzyme-linked immunosorbent assay tests also showed a significant reduction in total Aβ and Aβ40 or Aβ42 levels in Tris-buffered saline, SDS and formic acid fractions of brain homogenates and serum in both the prevention and treatment groups (Figure 3h, Supplementary Figure 11).

To investigate the specificity of ECD-Fc on amyloid burden, we performed additional experiments by injection of recombinant ECD-Fc or two control proteins human IgG or recombinant human

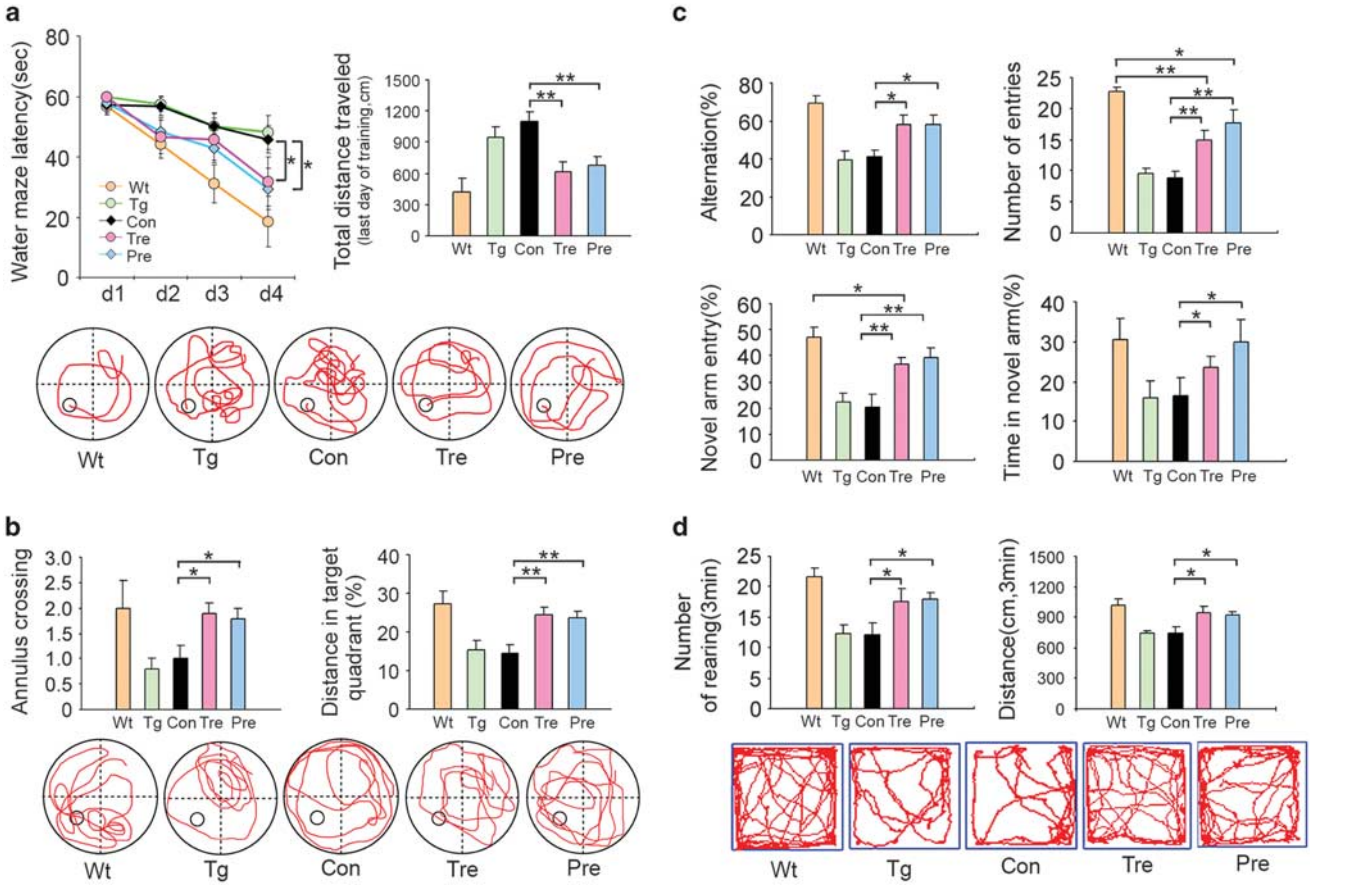
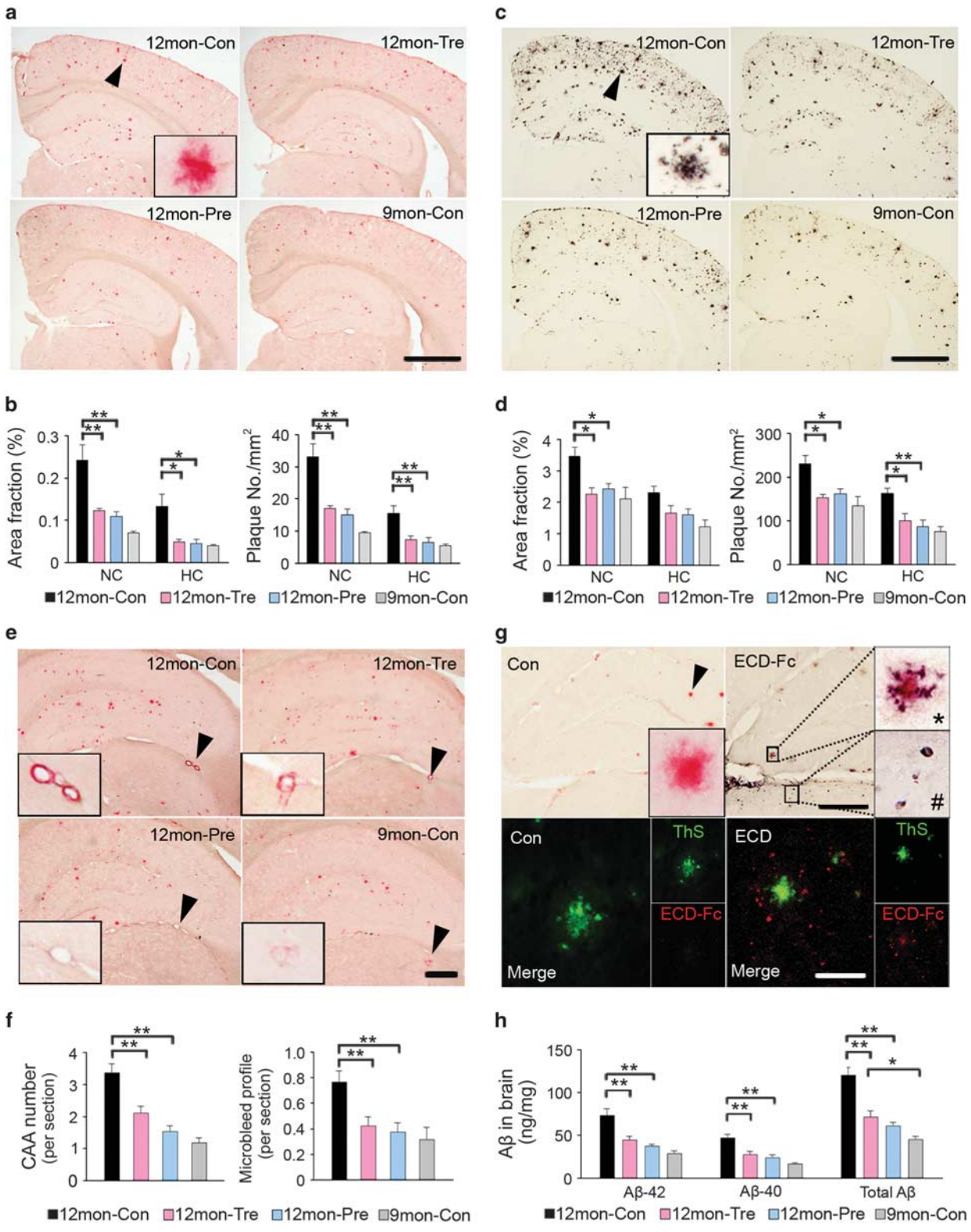


Figure 2. Protective effects of ectodomain-Fc in Alzheimer's disease mice at 12 months of age on memory and behavior. Mice were subjected to behavioral tests at 12 months of age, including wild-type littermates (wild type, $n = 11$), untreated mice with Alzheimer's disease (Tg, $n = 11$), mice with Alzheimer's disease treated with Adeno-associated virus-GFP as control (Con, $n = 14$), mice with Alzheimer's disease treated with Adeno-associated virus-ectodomain-Fc at 9 months of age for treatment (Tre, $n = 12$) and mice with Alzheimer's disease treated with Adeno-associated virus-ectodomain-Fc at 3 months of age for prevention (Pre, $n = 11$). (**a**) Escape latency (seconds) during platform trials and distance traveled (centimeters) on the last day of training in Morris Water Maze test (mean ± s.e.m., two-way analysis of variance, Tukey's test, $^*P < 0.05$, $^{**}P < 0.01$). (**b**) Number of crossings over the annulus of the platform in probe trial of Morris Water Maze test at day 5 (mean ± s.e.m.; one-way analysis of variance, Tukey's test, $^*P < 0.05$, $^{**}P < 0.01$). (**c**) Alternation of arms and numbers of entries in the spontaneous alternation test, and the number and time of entries in the novel arm test of Y-maze test (mean ± s.e.m.; one-way analysis of variance, Tukey's test, $^*P < 0.05$, $^{**}P < 0.01$). (**d**) The number of rearing and traveling distance in open-field test (mean ± s.e.m.; one-way analysis of variance, Tukey's test, $^*P < 0.05$). Representative locomotor activity tracing graphs are presented in the bottom panels.

Figure 3. Adeno-associated virus-ECD-Fc treatment reduces amyloid-beta levels in the brain of mice with Alzheimer's disease at 12 months of age. (**a**) Compact amyloid-beta plaques were visualized with Congo red. Inset shows the representative morphology of Congo red-positive plaque at higher magnification. Scale bar = 1mm. (**b**) Comparison of area fraction and plaque density of Congo red-stained plaques among groups. (**c**) The total amyloid-beta plaques stained with antibody 6E10 immunohistochemistry. Inset shows the representative morphology of 6E10-positive plaques at higher magnification. Scale bar = 1 mm. (**d**) Comparison of area fraction and density of 6E10-positive plaques among groups. (**e**) Cerebral amyloid angiopathy was visualized with Congo red staining. Inset shows the morphology of cerebral amyloid angiopathy at higher magnification. Scale bar = 1 mm. (**f**) Comparison of cerebral amyloid angiopathy and cerebral microbleed profiles among groups. (**g**) Co-localization of amyloid-beta plaque and ectodomain-Fc detected with amyloid-beta plaque staining (Congo red staining for top panels and Thioflavin S for bottom panels) and anti-human IgG Fc antibody. Scale bar: 200 μm (black), 40 μm (white). *shows co-localization of ectodomain-Fc with amyloid-beta plaques; # shows ectodomain-Fc expression in neurons. (**h**) Enzyme-linked immunosorbent assay analyses of Aβ40, Aβ42 and total Aβ of brain homogenates. 12mon-Con represents 12-month-old mice with Alzheimer's disease treated with Adeno-associated virus-GFP ($n = 11$); 12mon-Tre represents 12-month-old mice with Alzheimer's disease treated with Adeno-associated virus-ectodomain-Fc at 9 months of age ($n = 9$); 12mon-Pre represents 12-month-old mice with Alzheimer's disease treated with Adeno-associated virus-ectodomain-Fc at 3 months of age ($n = 8$); 9mon-Con represents 9-month-old mice with Alzheimer's disease as baseline control for 12mon-Tre group ($n = 7$). $^*P < 0.05$, $^{**}P < 0.01$ (mean ± s.e.m., one-way analysis of variance, Tukey's test).



fibroblast growth factor receptor 4 ECD fused with human IgG Fc (FGFR4-Fc) in the hippocampus of 8-months-old AD mice. A single injection of the recombinant ECD-Fc, but not human IgG or FGFR4-Fc, also significantly reduced the area fraction of amyloid plaques on the injected site 1 week after injection (Supplementary Figure 12). To investigate potential mechanism of how ECD-Fc affects the amyloid burden in the brain, we investigated the effect of ECD-Fc on Aβ fibrillation and disaggregation with ThT tests and transmission electronic microscopy visualization. Our data showed that ECD-Fc, but not the control proteins human IgG or FGFR4-Fc,

suppressed the Aβ fluorescence in fibrillation and the fluorescence of preformed Aβ fibers (Supplementary Figures 13a and b). The transmission electronic microscopy visualization showed that ECD-Fc, but not human IgG or FGFR4-Fc, prevented the formation of Aβ fibers and disaggregated the preformed Aβ fibers (Supplementary Figures 13c and d). Our data suggest that the effect of ECD-Fc on amyloid burden *in vivo* is likely a specific event not because of the nonspecific protein–Aβ interaction.

Restoration of brain p75ECD levels reduces amyloidogenic processing of APP by inhibiting BACE1 expression in AD mice

In sporadic AD, Aβ upregulates BACE1 expression and drives the vicious cycle leading to AD pathogenesis.[33–35] However, it is not known which receptor mediates the Aβ-JNK-BACE1 vicious cycle. We and others suggested that p75NTR may upregulate Aβ production,[29,36] forming a positive feedback. Thus, we investigated whether the disruption of the Aβ-p75NTR interaction with ECD-Fc can affect APP processing and Aβ production. The ECD-Fc treatment significantly reduced BACE1 expression and activity, and β-cleavage product (CTFβ) of APP and Aβ production in both prevention and treatment groups (Figures 4a and c). The level of production of the Aβ degrading enzymes, neprilysin and insulin degrading enzyme and the Aβ blood–brain barrier-transporting molecules, low-density lipoprotein receptor-related protein and receptor for advanced glycation end products, did not change (Supplementary Figure 14). To determine the mechanism by which ECD-Fc suppresses amyloidogenesis *in vivo*, we examined the expression of BACE1 and APP metabolites of BACE1 activity in primary neurons. We found that BACE1 expression and the sAPPβ level in the cultured primary neurons dose-dependently increased in response to Aβ42. This increase was abolished by the presence of recombinant ECD-Fc or in p75NTR knockout neurons (Figures 4d and e). This suggests that p75NTR is required for amyloidogenic APP processing, promoted by Aβ. These results indicate that p75NTR is a key receptor in mediating Aβ-induced BACE1 upregulation and has a critical role in the Aβ-BACE1 vicious cycle, driving the pathogenesis of AD and ECD-Fc treatment could break this vicious cycle.

Restoration of brain p75ECD levels attenuates neurite degeneration, neuronal death and Tau phosphorylation in AD mice

Compared with AAV-GFP-treated controls, the fractional areas stained for NeuN (neurons), MAP-2 dendrites and anti-choline acetyltransfetase positive axons in the hippocampus were increased in both prevention and treatment groups (Figure 5a), indicating that the ECD-Fc treatment can preserve neuronal structure in the hippocampus of AD mice. The number of dendritic spines detected by the Golgi staining in the hippocampus was significantly increased in both prevention and treatment groups (Figure 5b). The levels of synaptic proteins, including the major synaptic vesicle protein p38 synaptophysin, the vesicle-associated membrane protein 1, synaptosomal associated protein 25, postsynaptic density protein 95 and synapsin I were increased significantly in the brain of both prevention and treatment groups (Figure 5c). The fraction area of activated caspase-3 and terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate-biotin nick end labeling (TUNEL) staining, which identified apoptotic cells in the hippocampus, was significantly lower in the prevention and treatment groups compared with the control (Figure 5d). Consistent with the *in vivo* data, the recombinant p75ECD-Fc, but not human IgG and recombinant FGFR4-Fc (Supplementary Figure 15), suppressed the cell death of SH-SY5Y cells and protected cortical neurons from the neurite collapse triggered by Aβ *in vitro*.

The fraction area of Tau-phospho-Ser396 positive neurons in the hippocampus was lower in both the prevention and treatment groups compared with the control. The levels of Tau phosphorylation at multiple sites including serine 396, 262, 199 and threonine 231 were consistently and significantly diminished in the brain of both prevention and treatment groups (Figures 5e and h). We reason that the effect of p75ECD on Tau phosphorylation occurs via blocking Aβ which activates upstream GSK3β.[37] Indeed, we found that both the recombinant ECD-Fc and p75NTR neutralizing antibodies could block the increased phosphorylation of Ser262 of Tau in response to Aβ in the human SH-SY5Y cell line (Supplementary Figure 16). The recombinant ECD-Fc dose-dependently suppressed the phosphorylation of Ser262-Tau and the activity of GSK3β, which were elevated after Aβ42 treatment (Supplementary Figures 16b and c). This finding suggests that the elevation of p75ECD levels can protect against Aβ-induced axon and neurite degeneration, neuronal death and Tau hyperphosphorylation.

Restoration of brain p75ECD levels attenuates microgliosis, astrocytosis and inflammation in the brain of AD mice

Brain inflammation including microgliosis and astrocytosis is the secondary hallmarks of AD pathology. The fraction area of CD45 (microgliosis) and GFAP (astrocytosis) staining in the neocortex and hippocampus were significantly reduced in both prevention and treatment groups (Supplementary Figure 17). The levels of pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin (IL)-1β and IL-6 in the Tris-buffered saline fraction and serum were also lower in both prevention and treatment groups compared with the control group (Supplementary Figure 18). Improved performance in learning and memory tasks and reduced neuroinflammation are probable outcomes, secondary to the reduction in amyloid burden, Tau phosphorylation and neurodegeneration after ECD-Fc treatment.

Delivery of the ECD-Fc gene using AAV8 is well tolerated

Throughout the animal studies, no animal deaths had occurred and no obvious abnormal behavior was observed. The gene delivery did not significantly influence animal body weight or liver enzyme activities (Supplementary Figure 19a). No discernible pathological morphology was observed in major organs including the brain, muscle, heart, liver, lung, kidney and intestine (Supplementary Figure 19b). This data suggests that long-term expression of the ECD-Fc fusion gene in the brain is well tolerated by AD mice, demonstrating the safety of AAV-ECD-Fc treatment.

DISCUSSION

In the present study, we discovered that soluble p75ECD is a potentially useful therapeutic and biomarker for AD. Soluble p75ECD is physiologically distributed in central nervous system and is developmentally regulated by ageing. The p75ECD level is significantly reduced in the brain of AD subjects and mice, likely because of the Aβ-induced reduction in the expression and activity of TACE. p75ECD protects neurons from Aβ-induced neurotoxicity and suppresses BACE1 expression and Tau phosphorylation. The restoration of p75ECD levels in the brain of AD mice protects the brain from neurodegeneration. In AD animals, brain p75ECD levels are negatively correlated with cognitive impairment, brain Aβ burden and inflammation (Supplementary Table 1). Together with the preliminary finding that p75NTR SNP rs2072446 (S205L mutation) is significantly associated with sporadic AD in a Chinese population,[38] our data in the present study indicate that p75 plays a critical role in AD pathogenesis.

The source of p75ECD in CSF originates from p75NTR-expressing cells in the brain and spinal cord. Normally in the adult CNS, p75NTR is expressed in cholinergic neurons in the basal forebrain,[29,39] Purkinje and granule neurons in the cerebellum,[40]

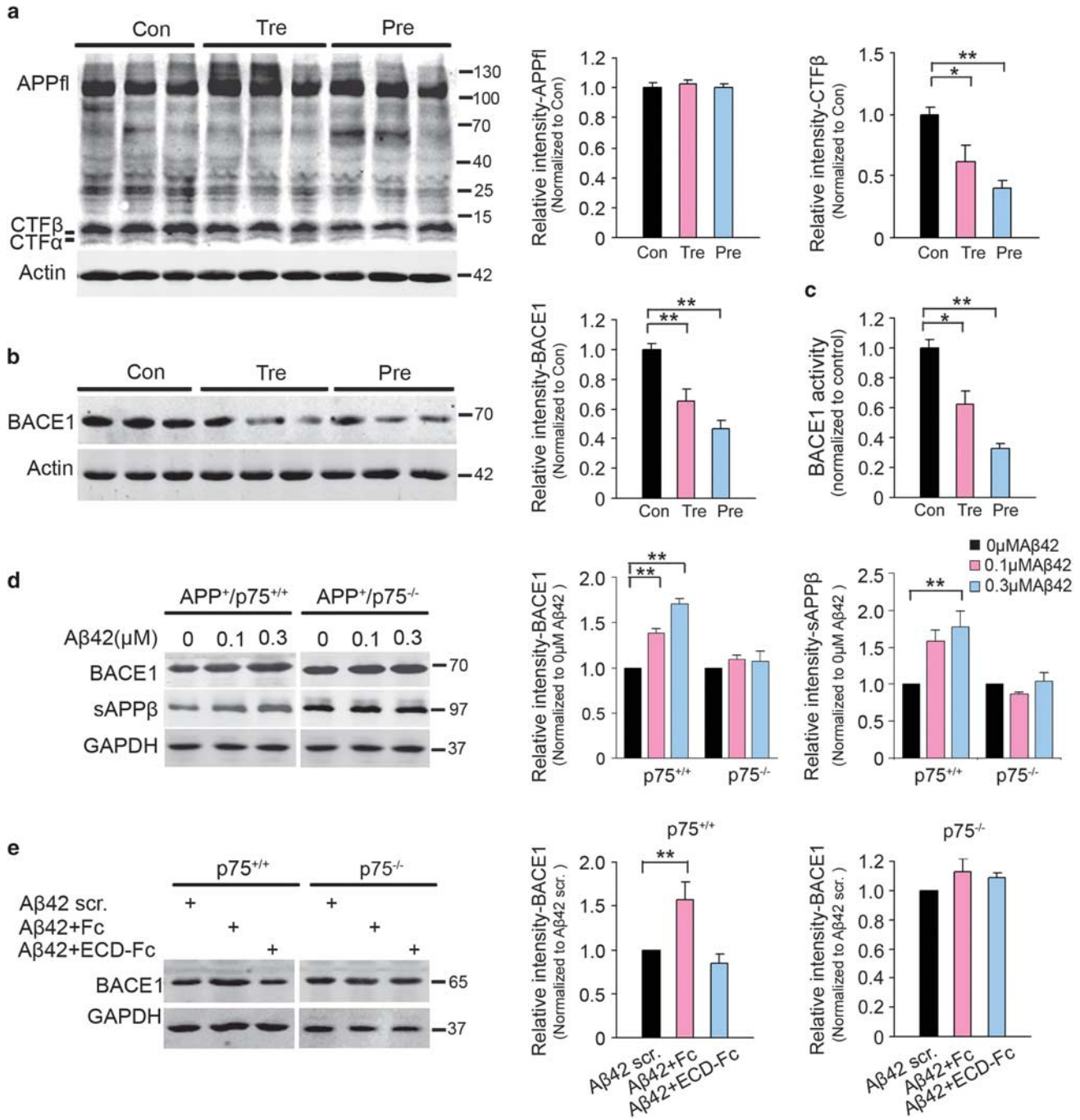


Figure 4. ECD-Fc treatment reduces amyloid-beta production via inhibition of β-site APP-cleaving enzyme expression. (**a**) Western blot of full length amyloid precursor protein and carboxyl-terminal fragments in brain lysates ($n = 8$, mean ± s.e.m., analysis of variance, Tukey's test, $^{**}P < 0.01$). (**b**) Western blot of β-site APP-cleaving enzyme in brain lysates ($n = 8$, mean ± s.e.m., analysis of variance, Tukey's test, $^{**}P < 0.01$). (**c**) Analysis of β-site APP-cleaving enzyme activity in the mouse brain ($n = 11$ for Con, $n = 9$ for Tre, $n = 8$ for Pre, mean ± s.e.m., analysis of variance, Tukey's test, $^{**}P < 0.01$). (**d**) Western blot of β-site APP-cleaving enzyme and sAPPβ in the extracts of cultured primary cortical neurons from mice with Alzheimer's disease with (AD/p75$^{+/+}$) or without p75NTR gene (AD/p75$^{-/-}$) after treatment with different doses of Aβ42 ($n = 3$ per dose, mean ± s.e.m., analysis of variance, Tukey's test, $^{**}P < 0.01$). (**e**) Western blot of β-site APP-cleaving enzyme in extracts of cultured primary cortical neurons from wild-type mice (p75$^{+/+}$) or p75NTR knockout mice (p75$^{-/-}$) after treatment with 0.3 μm Aβ42 in presence or absence of recombinant ECD-Fc ($n = 3$ per dose, mean ± s.e.m., analysis of variance, Tukey's test, $^{*}P < 0.05$). scr., scramble control.

sensory and motor neurons in the brain stem and spinal cord,[41] and oligodendrocyte precursors.[42] p75NTR is upregulated in response to pathological stimuli such as neurotrauma, inflammation and epilepsy.[8,20,41] Although FL p75NTR is a death receptor that regulates apoptosis and degeneration during development and in pathological conditions,[8,20] the function of soluble p75ECD after shedding remains unknown. Whether the change of p75ECD in the CSF and brain is specific in AD is not known. Future

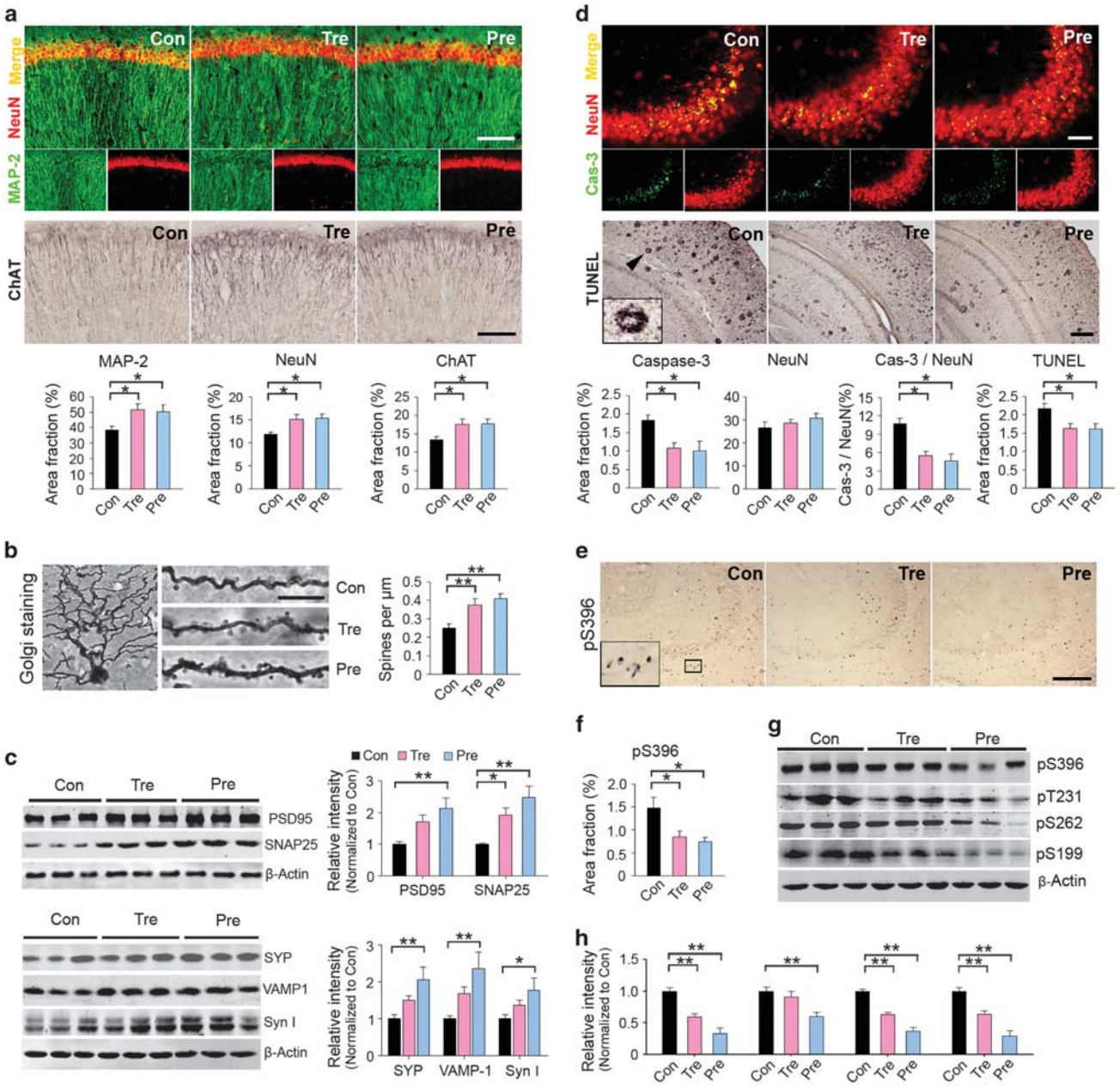


Figure 5. p75NTR ectodomain antagonizes amyloid-beta toxicity in mice with Alzheimer's disease at 12 months of age. (**a**) The overall preserved structure of neurons and dendrites was quantified using anti-MAP-2 and anti-NeuN immunofluorescence or anti-choline acetyltransfetase immunohistochemistry ($n = 11$ for Con, $n = 9$ for Tre, $n = 8$ for Pre, mean ± s.e.m., one-way analysis of variance, Tukey's test, $^*P < 0.05$, $^{**}P < 0.01$). Scale bar = 100 μm. (**b**) Representative photomicrograph of a whole Golgi-stained CA1 hippocampal neuron (left) and comparison of dendritic spines intensity of basal segment of CA1 hippocampal neuron among groups (bar = 10 μm, three mice per group and seven neurons per mouse, mean ± s.e.m., one-way analysis of variance, Tukey's test, $^*P < 0.05$). (**c**) Western blot analyses of synapse-associated proteins including synaptosomal associated protein 25, postsynaptic density protein 95, synapsin I, vesicle-associated membrane protein 1 and synaptophysin ($n = 8$, mean ± s.e.m., one-way analysis of variance, Tukey's test, $^*P < 0.05$, $^{**}P < 0.01$). (**d**) Neuronal apoptosis was visualized by using activated caspase-3 (cas-3) immunofluorescence (scale bar = 50 μm) and terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate-biotin nick end labeling (TUNEL) staining (scale bar = 250 μm, $n = 8$ for Con, $n = 9$ for Tre, $n = 8$ for Pre, mean ± s.e.m., one-way analysis of variance, Tukey's test, $^*P < 0.05$, $^{**}P < 0.01$). (**e** and **f**) Tau phosphorylation immunostaining in hippocampus by using pSer396 antibody (Scale bar = 200 μm). (**g** and **h**) Western blot analyses of Tau-hyperphosphorylation at multiple sites including serine 199, 262, 396 and threonine 231 ($n = 8$ for each group, mean ± s.e.m., one-way analysis of variance, Tukey's test, $^*P < 0.05$, $^{**}P < 0.01$).

investigations on p75ECD and p75FL in the CSF and brains of other neurological disorders are required to validate whether p75ECD and p75FL are specific diagnostic markers for AD. We have discovered that p75ECD is a neuroprotective molecule which protects neurons against neurodegeneration in the AD brain. FL p75NTR and neurodegeneration-causing ligands, Aβ and proNGF, are all upregulated,[11–14,36,43,44] while BDNF/trkB signaling is downregulated in sporadic AD brains.[6,7] These changes lead to an imbalance between neurotrophic and neurodegenerative signaling in sporadic AD.[45] As neurodegenerative factors, Aβ

and proNGF, activate FL p75NTR by binding the ectodomain to trigger neurodegenerative signaling, the soluble p75ECD after shedding can scavenge for Aβ and other neurodegenerative factors. Indeed, soluble p75ECD is clearly demonstrated to be neuroprotective in our previous and current *in vitro* and *in vivo* studies.[46,47] Thus, the cleavage of the ectodomain of p75NTR becomes a critical event of switching the neurotoxic signaling of Aβ and proNGF to the neuroprotective effects of p75ECD. Surprisingly, the p75ECD level is significantly reduced in AD, suggesting the cleavage process in AD is impaired by Aβ. As TACE is a physiological sheddase which regulates the production of p75ECD,[26,28] the reduced level of p75ECD in the AD brain is a likely result of the impaired p75NTR cleavage due to the reduction in the expression and activity of TACE, triggered by Aβ. It is known that the prion protein and Aβ can suppress TACE activity by activating PDK1 in models of prion disease and AD.[48]

In the AD brain, Aβ triggers upregulation of BACE1 expression which promotes Aβ generation, creating a vicious cycle and resulting in Aβ overproduction.[33–35] Aβ is both an initiating factor and a product of this vicious cycle. However, the mechanism underlying the cycle is not clear. We have discovered that p75NTR mediates Aβ-induced BACE1 upregulation, as both ECD-Fc and p75NTR knockout can block Aβ-induced BACE1 upregulation and inhibit amyloidogenic APP processing. Our data suggests that FL p75NTR is a central player in driving the Aβ-BACE cycle. The reduction of p75ECD levels in the AD brain may contribute to the overproduction of Aβ, exacerbating this vicious cycle. However, restoring p75ECD levels *in vivo* can suppress BACE1 expression and the associated amyloidogenic pathway. Furthermore, p75ECD can break the Aβ-BACE1 vicious cycle, which contributes to Aβ overproduction in sporadic AD.

p75ECD protects neurons at multiple levels of the Aβ cascade leading to the pathogenesis of AD, including the suppression of Aβ aggregation and deposition,[29] Aβ overproduction and neurotoxicity, and Aβ-induced Tau phosphorylation (Supplementary Figure 20). The treatment with ECD-Fc and p75NTR antibodies *in vitro* and *in vivo* can block the Tau phosphorylation, suggesting that Aβ-induced Tau phosphorylation is likely mediated by p75NTR. p75NTR also mediates apoptosis and degeneration induced by proneurotrophins,[20] and p75ECD can block neurite collapse mediated by proNGF and proBDNF.[46,47] Therefore, it is conceivable to assert that soluble p75ECD interacts with Aβ and proneurotrophins, and acts as a neurotoxin scavenger to block their signaling through FL p75NTR.[47] In addition, Aβ deposition accelerates in AD brain after 9 months of age (Figure 3), suggesting that there is an endogenous mechanism which protects against Aβ deposition. Our present study reveals that p75ECD is a novel endogenous anti-Aβ deposition molecule which is compromised in the AD brain. In summary, we have uncovered a novel mechanism of dysfunctional shedding of p75NTR in AD and p75ECD is a neuroprotective molecule, which protects the brain from Aβ production, deposition and toxicity. Based on the failure of current clinical trials of immunotherapies and secretase inhibitors for AD, we have proposed that therapeutic reagents targeting multiple aspects of AD pathogenesis are needed for the successful management of AD.[49] Owing to the multiple protective effects and reduced levels of p75ECD in the brain of AD, the restoration of p75ECD levels in the brain could be a desirable therapeutic approach for AD.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

YJW and XFZ conceived and designed the project and wrote the manuscript, XQY, SSJ, FZ, QHW, CRL, CZ, LLS, GHZ, YHL and HDZ performed *in vivo* experiments and analyzed data, KS, JW, YQZ and JHZ performed *in vitro* experiments and analyzed data, LFL, DGW, HYH, BG and JT analyzed human brain samples and revised manuscript.

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