Increased Expression of PS1 Is Sufficient to Elevate the Level and Activity of γ-Secretase In Vivo

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Abstract

Increase in the generation and deposition of amyloid-β (Aβ) plays a central role in the development of Alzheimer’s Disease (AD). Elevation of the activity of γ-secretase, a key enzyme required for the generation of Aβ, can thus be a potential risk factor in AD. However, it is not known whether γ-secretase can be upregulated in vivo. While in vitro studies showed that expression of all four components of γ-secretase (Nicastrin, Presenilin, Pen-2 and Aph-1) are required for upregulation of γ-secretase, it remains to be established as to whether this is true in vivo. To investigate whether overexpressing a single component of the γ-secretase complex is sufficient to elevate its level and activity in the brain, we analyzed transgenic mice expressing either wild type or familial AD (fAD) associated mutant PS1. In contrast to cell culture studies, overexpression of either wild type or mutant PS1 is sufficient to increase levels of Nicastrin and Pen-2, and elevate the level of active γ-secretase complex, enzymatic activity of γ-secretase and the deposition of Aβ in brains of mice. Importantly, γ-secretase comprised of mutant PS1 is less active than that of wild type PS1-containing γ-secretase; however, γ-secretase comprised of mutant PS1 cleaves at the Aβ42 site of APP-CTFs more efficiently than at the Aβ40 site, resulting in greater accumulation of Aβ deposits in the brain. Our data suggest that whereas fAD-linked PS1 mutants cause early onset disease, upregulation of PS1/γ-secretase activity may be a risk factor for late onset sporadic AD.

Introduction

Alzheimer’s disease (AD), the most common form of dementia occurring in the elderly, is a progressive neurodegenerative disease characterized pathologically by neuritic (or senile) amyloid plaques and neurofibrillary tangles in the brain [1]. Neuritic plaques are mainly consists of amyloid-β deposits in the brain [1]. Neuritic plaques are characterized pathologically by neuritic (or senile) amyloid plaques and neurofibrillary tangles [1]. Neuritic plaques are characterized pathologically by neuritic (or senile) amyloid plaques and neurofibrillary aggregates of Aβ, which are neurotoxic and initiate a cascade of events eventually leading to synaptic and neuronal dysfunction and death in cases of AD. This view is supported by the genetic studies in which missense mutations in APP [3–5], PS1 and PS2 [6–9], and duplications of APP [10] have been linked to cases of early onset familial AD (fAD). All of these mutations either increase the generation of Aβ or increase relative levels of Aβ42, the more toxic form of Aβ. As only symptomatic therapies are currently available, mechanism-based disease modifying therapy remains a major unmet need for AD[11].

FAD accounts for ~5% of total AD cases, whereas >90% of individuals with AD manifest as late onset sporadic form of AD (sAD). While the genetic risk factors that contribute to Aβ amyloidosis in sAD cases are less clear, putative sporadic cases are influenced by a variety of susceptibility genes and possibly other less well-defined factors [12]. One gene that has been consistently replicated as a major dose-dependent risk factor in a large number of studies across many ethnic groups is the ApoE allele of the apolipoprotein E gene (chromosome 19q13), which has been hypothesized to influence Aβ metabolism, Aβ aggregation/degradation/clearance [13–15]. Recent research has identified gene variants encoding ubiquitin1 (UBQLN1) [16] and sortilin1 (SORL1) [17] as risk factors, and GWAS approaches have identified a number of variants (CLU, PICALM and CRI) associated with AD [18,19], which may also contribute to abnormal APP processing and Aβ accumulation. Therefore, it has been widely accepted that both sporadic and familial AD share the same underlying disease mechanism by promoting the accumulation of Aβ in the brain.

As enzymes required for the generation of Aβ, increase of β- and γ-secretase activities in the brain represent potential risks for the development of AD. While it has been reported that higher levels of BACE1 are associated with increased risk for AD [20,21], less information is available regarding the relationships between altered levels of γ-secretase and risk of development of AD.
Moreover, the idea that γ-secretase is a risk factor for AD is challenged by the assumption that upregulation of γ-secretase is difficult to achieve. Although PS alone exhibits γ-secretase activity, each component of the γ-secretase complex, PS, Nct, APH-1 and PEN-2, is absolutely required for γ-secretase activity in vitro [22]. While the mechanism of assembly of γ-secretase complex is still not completely understood, the accumulation of γ-secretase appears to be tightly controlled. A simple model of γ-secretase assembly invokes initially the formation of a relatively stable pre-complex comprised of Nct and Aph-1 [23], and the subsequent associations of PS and PEN-2 with this pre-complex lead to the formation of the mature γ-secretase complex. The mature γ-secretase complex is characterized by the post-translational modifications of various subunits, while the enzyme complex is transported en route to the plasma membrane. Evidence indicates that some components of γ-secretase complex, such as PS, are not stable unless they are associated with other components to form the pre- or mature γ-secretase complex [23–26]. Therefore, the downregulation of γ-secretase can be achieved by reducing anyone of the four essential components of γ-secretase.

In contrast, overexpression of any one member has little effect on the levels of other components or the overall γ-secretase activity in cell culture systems [27–31]. In fact, overexpression of even any combination of three proteins is still not sufficient to increase the activity of γ-secretase; elevation in γ-secretase activity can only be achieved by overexpressing all four proteins [32]. These observations led to the assumption that the upregulation of γ-secretase is unlikely to occur in the brain since upregulation of all four components of γ-secretase is necessary to increase the level of γ-secretase. However, this assumption is based on in vitro cell culture studies and has not been thoroughly tested in vivo.

In this report, we document that, in contrast to the observations derived from cell culture studies, increased expression of PS1 alone significantly increased the level and activity of γ-secretase and the Aβ burden in the brain, suggesting the possibility that elevation of γ-secretase may be a risk factor in AD.

**Results**

While previous efforts demonstrated that increased expression of all four components of γ-secretase is required to upregulate its enzymatic activity in cultured cells, it remains to be established whether overexpression of all four components is necessary to increase the level of γ-secretase in vivo. To determine whether overexpression of a single component of the γ-secretase is sufficient to elevate its level and activity in the brain, we analyzed transgenic mice expressing human wild type PS1 under the control of mouse Ptp promoter [33]. Initial protein blot analysis of levels of PS1 in brains of PS1 transgenic mice using antibodies specific to C-terminal fragments of PS1 (one of two processed fragments of PS1 associated with the mature γ-secretase complex), revealed overexpression of human PS1 protein (Fig. 1A). To confirm the “replacement effect” of the exogenous human PS1, we assessed the level of endogenous PS by analyzing the level of PS2 using antiserum recognizing the C-terminal fragment of PS2 (PS2-CTF). As expected, the level of PS2-CTF was significantly reduced in brains of PS1 transgenic mice as compared to that of controls (Fig. 1A and 1B). Since anti-PS1 antibody can recognize the human and mouse PS1 with higher affinity, levels of γ-secretase complex in PS1 transgenic mice cannot be evaluated by Western blot using antibodies against PS1. To assess the levels of γ-secretase complex, we analyzed the endogenous protein levels of Nct and PEN2, two key components of γ-secretase complex in brains of PS1 transgenic mice. Interestingly, protein levels of PEN2 was significantly increased (~200%, p<0.005) in PS1 transgenic mice as compared to that of wild type mice (Fig. 1A and 1B). Consistent with this finding, we also observed an increase of endogenous Nct in PS1 transgenic mice as compared to that of wild type mice (Fig. 1A and 1B).

To determine whether the γ-secretase complex in PS1 transgenic mice is functional and active, we used an in vitro γ-secretase activity assay to assess the γ-secretase enzymatic activity in protein extracts derived from brains of PS1 transgenic mice. Interestingly, γ-secretase activity at both the Aβ40 (Fig 1C) and Aβ42 cleavage site in brain extracts of PS1 transgenic mice increased ~150% as compared to control mice, corroborating the increase in protein levels of components of the γ-secretase complex observed in the brains. Consistent with our previous studies [34,35], γ-secretase activities in brains of Nct<sup>+/-</sup> mice were reduced ~50% as compared with that of wild type mice (Fig. 1C). These results establish that elevated levels of PS1 alone in the brain of mice is sufficient to increase the level and enzymatic activity of mature γ-secretase complex in vivo.

While both PS1 and PS2 can form functional γ-secretase complex, it has been reported that the specific activity of PS1-containing γ-secretase is higher than that of PS2-containing complex in vitro [36]. Exogenous expression of human PS1 replaces both the endogenous PS1 and PS2, thus most γ-secretase in the PS1<sup>wt</sup> transgenic mice are human PS1 containing γ-secretase complex. While we observed a significant increase of γ-secretase activity in PS1<sup>wt</sup> mice, it is possible that the increased γ-secretase activity in PS1<sup>wt</sup> transgenic mice is due to relative abundance of PS1-containing γ-secretase complex. To examine whether overexpression of PS1 can increase the levels of active γ-secretase complex, we used a well characterized γ-secretase transition-state analogue inhibitor based probe (compound 4) [37] to quantify the active fraction of γ-secretase in vivo. Biotin labeled compound 4 can specifically bind to the active γ-secretase complex, thus active γ-secretase can be captured using streptavidin-labeled bead [37]. Membrane proteins were extracted using buffer containing CHAPSO and the levels of PS1 and Nct in unbounded fractions and affinity captured fractions of membrane protein extracts were subjected to immunoblot analysis using antisera against Nct, PS1-NTF and PS1-CTF. We found that ~70% of CHAPSO extracted γ-secretase complex can be captured by the compound-4, while another membrane-bound secretase, BACE1, was not captured by compound 4 (Fig 1D), which confirmed that compound 4 is a specific and high affinity inhibitor of γ-secretase. Comparing the levels of Nct and PS1 in the affinity captured fraction of PS1<sup>wt</sup> transgenic with that of non-transgenic control mice, we found that the levels of Nct in affinity-captured fraction of PS1<sup>wt</sup> mice increased by more than 130% (Fig. 1D and 1E). These data further support our view that overexpression of PS1 alone in the brain increase the protein level of mature γ-secretase complex and enzymatic activity of γ-secretase in vivo.

While our studies demonstrate that overexpression of PS1 can significantly increase the level of mature γ-secretase in the brain, it is not clear whether such an increase is derived from neuronal or non-neuronal cells. Since Aβ is generated from sequential cleavage of APP by β- and γ-secretase and β-secretase (BACE1) is abundantly expressed in neurons [38], the magnitude of Aβ secretion will be dependent on the level of γ-secretase in neuronal cells. To determine whether overexpression of PS1 can upregulate the level of γ-secretase in neurons, we isolated primary neuronal and non-neuronal cells from brains of PS1 transgenic and wild type embryos (at embryonic day 17) and assessed the levels of γ-secretase in these cells. The purity of our neuronal cultures was determined using the neuron specific marker, β-tubulin III.
Interestingly, in either the neuronal or non-neuronal cell cultures derived from PS1 transgenic mice, the levels of Nct were significantly increased (Fig. 2A and 2B), indicating that the overexpression of PS1 alone is sufficient to increase the level of γ-secretase in either neuron or non-neuronal cells.

To examine the effects of upregulation of γ-secretase in PS1 transgenic mice on generation of Aβ and Aβ amyloidosis, we crossed the PS1 mice with a mouse model of amyloidosis, APP<sup>swe</sup> transgenic mice [39], to generate APP<sup>swe</sup>;PS1<sup>wt</sup> mice. Although no Aβ deposits were detected in the brains of 9 month-old APP<sup>swe</sup>;PS1<sup>wt</sup> or APP<sup>swe</sup> mice, ELISA analysis showed an ~30% increase in the level of Aβ40 in brains of APP<sup>swe</sup>;PS1<sup>wt</sup> mice as compared to that of APP<sup>swe</sup> mice (Fig. 3A, p<0.05), indicating that upregulation of PS1 alone is sufficient to increase the generation of Aβ in brains of mice. To determine whether such increase in γ-secretase activity would accelerate amyloid deposition, serial brain sections of 22 month-old APP<sup>swe</sup>;PS1<sup>wt</sup> mice were stained with antibodies against Aβ (6E10) or against ubiquitin. Interestingly, greater amount of Aβ plaques were observed in brain sections of APP<sup>swe</sup>;PS1<sup>wt</sup> mice as compared to that of APP<sup>swe</sup> mice (Figs. 3D). Since the levels of APP in APP<sup>swe</sup>;PS1<sup>wt</sup> mice is identical to that of APP<sup>swe</sup> mice (Fig. 3E), the elevation of Aβ deposition is likely due to an increase in levels of γ-secretase in brains of PS1<sup>wt</sup> mice. To quantify the levels of Aβ deposition, we used both filter trap and unbiased stereology approaches to assess the amyloid burden in the brains of these transgenic mice. We observed not only an ~2 fold increase of aggregated Aβ (Fig. 3B and 3C), but importantly, a corresponding elevation of amount of Aβ plaques (Fig. 3F) in the brains of APP<sup>swe</sup>;PS1<sup>wt</sup> mice as compared to those of APP<sup>swe</sup> mice. These
results demonstrate that overexpressing PS1 alone is sufficient to increase the activity of γ-secretase and deposition of Aβ in the brain and thus suggest that such increase of γ-secretase activity may confer risk in development of AD.

To further establish that overexpression of PS1 alone could increase the level of γ-secretase in the brain, we examined the effects of overexpression of a variety of fAD-linked PS1 mutants in mice. We first examined the protein levels of γ-secretase in transgenic mice that overexpress PS1AE9 and PS1-A246E (Fig. 4A). Consistent with our observation in wild type PS1 transgenic mice, mice expressing PS1 mutants also exhibit significant increase in the level of γ-secretase complex in the brains. The levels of several components of γ-secretase (PS1, Nct, PEN2) in PS1AE9 were similar as compared with those of PS1 transgenic mice, while the levels of components in PS1-A246E mice were higher than those of PS1 transgenic mice (Fig. 4B), which is consistent with previous reports [40]. Surprisingly, the increase in levels of γ-secretase components in the brains of PS1AE9 and PS1-A246E mice did not lead to significant increase in cleavage activity at the Aβ40 site (Fig. 4C). Rather, the γ-secretase activity at the Aβ40 site in brains of PS1-A246E mice were 2–3 folds lower than that of wild type PS1 mice (Fig. 4C) and Aβ40 site cleavage activity observed in PS1AE9 mice was even lower than that of non-transgenic mice (Fig. 4C). In parallel, we assessed the cleavage activity at the Aβ42 site in brain extracts derived from these mice (Figure 4D). Interestingly, the activity at the Aβ42 site of γ-secretase in PS1-A246E mice was significantly higher than that of PS1 and non-transgenic mice (Fig. 4D). γ-Secretase activity in brains of PS1AE9 mice was similar to that of non-transgenic mice. These findings indicate that the γ-secretase complex containing mutant PS1 is less active than that comprised of wild type PS1, especially with respect to the cleavage activity at the Aβ40 site. However, whereas the ratio of Aβ40/Aβ42 activity in PS1 mice was identical to that of non-transgenic mice, this ratio was significantly decreased in both PS1AE9 and PS1-A246E mice (Fig. 4E). These observations are consistent with the view that γ-secretase containing fAD-linked PS1 mutants elevate the relative cleavage activity at the Aβ42 site as compared to the Aβ40 site [39].

To compare the effects of mutant PS1 on Aβ deposition with that of wild type PS1, we crossed PS1AE9 mice with the APP<sup>sw</sup> mice to generate PS1AE9;APP<sup>sw</sup> mice and assessed the Aβ amyloidosis in their brains. As anticipated, we observed that the Aβ burden in the brains of PS1AE9;APP<sup>sw</sup> mice was significantly higher than that of the APP<sup>sw</sup> mice or PS1<sup>wt</sup>/APP<sup>sw</sup> mice (Fig. 4F and 4G). These results are consistent with the idea that the relative ratio of Aβ42/Aβ40 is a critical determinant for Aβ deposition in the brain [39].

**Discussion**

Since γ-secretase is a key enzyme for the generation of Aβ peptides, understanding how γ-secretase contributes to the development of AD has been a major focus in the field. Mutations in PS1 and PS2 have been identified in some rare cases of early onset FAD [6–9]. These mutations increase the relative levels of Aβ42 [39], a more amyloidogenic and toxic form of Aβ. In contrast, that increases in level of γ-secretase accelerate the production of Aβ to elicit the Aβ associated pathology would provide a molecular mechanism for increasing the risk for AD. Duplications of APP [10] in cases of AD is an example of such a mechanism in which risk for AD is elevated through the increased production of Aβ. However, no genetic association between increased level of γ-secretase and higher risk of AD has been reported. Furthermore, it is unknown as to how expression and accumulation of γ-secretase is regulated in the brain. It is well recognized that the subunit assembly of γ-secretase complex is tightly regulated such that excess subunits like PS or PEN2, which are not recruited into stable complexes are rapidly degraded. Therefore, deletion of any component of the γ-secretase leads to the disassembly of the γ-secretase complex. In contrast, cell culture studies showing that levels of γ-secretase can only be elevated by increased expression of all four components has fueled the idea that γ-secretase would be more amenable to mechanisms that down-regulates its levels than those that up-regulates it. For example, a mutation that reduces expression of one of the four components is predicted to decrease the level of γ-secretase. However, even polymorphism occurring in three of the four genes

![Figure 2. Increase of γ-secretase in primary neurons and non-neuronal cells derived from PS1 mice.](image-url)
would still not be sufficient to increase \( \gamma \)-secretase activity, only variants occurring simultaneously in all four genes encoding components of the \( \gamma \)-secretase may up-regulate its activity. The realization that \( \gamma \)-secretase is an essential enzymes for not only the processing of APP, but also required for processing a growing list of type I transmembrane proteins in a process called "regulated intramembrane proteolysis" [41] indicates that maintenance of a stable level of \( \gamma \)-secretase is essential for the normal function of organisms. Surprisingly, the studies described herein demonstrate that increased expression of PS1 alone is sufficient to increase the \( \gamma \)-secretase activity and to elevate deposition of A\( \beta \) in vivo, indicating that alteration of a single component of \( \gamma \)-secretase is sufficient to elevate the level of \( \gamma \)-secretase. While the clinical relevance of our finding is still unclear, our observation also suggests that increase in levels of PS1 should have the most dramatic impact on the levels of \( \gamma \)-secretase in vivo.

While the increase of \( \gamma \)-secretase elevated the production of A\( \beta \) in mouse brains of PS1 mice, the amyloid burden was only modestly increased. In parallel, the level of \( \gamma \)-secretase in the IAD-linked mutants PS1-A246 and PS1-AE9 mice can also be increased. Interestingly, the \( \gamma \)-secretase complex containing PS1-A246E or PS1-AE9 mutant exhibited a drastic reduction in level of \( \gamma \)-secretase activity at the A\( \beta \)40 site as compare to that of wild type PS1 mice. Instead, relative levels for A\( \beta \)42 processing were increased as compared to that of the wild type PS1 mice whose A\( \beta \)42/40 ratio remained constant and consequently, accelerated the amyloid burden in brains of PS1-AE9 mice. Our findings are consistent with the notion that A\( \beta \)42 is more toxic and readily form A\( \beta \) aggregates in the brain [39].

Figure 3. Increase in generation and deposition A\( \beta \) in the brains of APP\textsuperscript{swe},PS1 mice. (A) ELISA analysis of A\( \beta \)40 peptides in the protein extracts of brains of PS1 and non-transgenic control mice. The data were average +/- SEM from 5 mice for each genotype. (B) Sagittal brain sections (10 \( \mu \)m) of hippocampus area of 22-months old APP\textsuperscript{swe},PS1 and APP\textsuperscript{swe} female mice. The A\( \beta \) plaques were visualized by immunostaining with antibodies specific to ubiquitin and A\( \beta \) peptides (6E10). (C) Quantitative analysis of the levels of A\( \beta \) aggregation in the brains of APP\textsuperscript{swe},PS1 and APP\textsuperscript{swe} mice at 22 months of age by filter trap assay. (D) Quantification of the signals of A\( \beta \) aggregations in the filter trap assay using Image J program. (E) Analysis of A\( \beta \) deposition using unbiased stereology in the hippocampus of 22-months old APP\textsuperscript{swe},PS1 (\( n = 6 \)) and APP\textsuperscript{swe} (\( n = 10 \)) female mice. doi:10.1371/journal.pone.0028179.g003
Figure 4. Overexpression of FAD linked PS1 mutants increases the level of γ-secretase in the brain. (A) Protein extracts (20 μg each) from brains of PS1ΔE9 (lanes 1, 2), PS1-A246E (lanes 3, 4), PS1wt (lanes 5, 6) and non-transgenic mice (lanes 7–9) were immunoblotted with anti-sera specific to Nct, PS1-NTF, PS1-CTF, PEN2, SOD-1 or Actin. (B) Quantification of signals of Nct, PEN2 and SOD-1 in protein blots of mutant and wild type PS1 and non-transgenic control mice by Image J program. The signal density was normalized using Actin signal derived from the same blot. (C) In vitro γ-secretase assay of Aβ40 cleavage in brain extracts of PS1ΔE9, PS1-A246E, PS1wt and non-transgenic control mice. The data represent averages +/- SEM from 4 mice for each genotype. (D) In vitro γ-secretase assay of Aβ42 cleavage in brain extracts of PS1ΔE9, PS1-A246E, PS1wt and non-transgenic control mice. The data represent averages +/- SEM from 4 mice for each genotype. (E) The ratio of Aβ40 and Aβ42 cleavage activity in brain extracts.
of PS1E9, PS1-A246E, PS1wt and non-transgenic control mice. (F) Sagittal brain sections (10 µm) of 22-months old APPswe;PS1E9 female mice. The Aβ plaques were visualized by immunostaining with antibodies specific to ubiquitin and Aβ peptides (6E10). (G) Analysis of Aβ deposition using unbiased stereology in brain sections of 22-months old APPswe;PS1E9 (n = 5), APPswe;PS1 (n = 6), and APPswe (n = 10) female mice. The data represent averages +/- SEM from each genotype.

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In summary, our data support the view that overexpression of PS1 in vivo is sufficient to increase the generation and deposition of Aβ in the brain. Importantly, this unexpected finding strongly implicates that upregulation of γ-secretase could contribute to increased risk of AD, especially in late onset sporadic AD.

Materials and Methods

Animals and cell lines

PS1(wt), PS1E9, PS1A246E, and APPswe mice were generated as described previously [33,45]. PS1(wt) and PS1E9, mice were crossed with APPswe mice to generate PS1wtAPPswe and PS1E9APPswe mice. Animal housing and experimental protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University (Protocol # MO09M243).

Affinity Capture of Endogenous γ-Secretase

The affinity capture of active γ-secretase was performed using a procedure modified from that described previously [37]. In short, 100 µg CHAPSO-solubilized membrane proteins were incubated for 2 h at 37°C in 50 mM PIPES, pH 7.0, 150 mM KCl, 5 mM CaCl₂, 5 mM MgCl₂, and protease inhibitors in 0.5% (v/v) CHAPSO with 20 mM biotinylated affinity probe (compound 4), which is modified from a potent transition state analog of γ-secretase (L458) [37]. 250 µl [10 mg/ml] of streptavidin-coupled magnetic beads (Dynal, Invitrogen) were added to the reaction and incubated overnight at 4°C. Captured complexes were washed five times with TBST buffer (Tris-buffered saline containing 0.1% (v/v) Tween 20) and eluted with 2X SDS sample buffer. Samples were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. PS1 and Nct were detected by protein blotting using antisera against PS1-NTF, PS1-RNF, and Nct. The levels of BACE1 served as a loading control. In all cases, protein blots shown are representatives of three or more experiments.

Primary Cortical Neuronal and Non-neuronal Cells

Mouse primary neuronal cultures were obtained from cerebral cortices of E18 embryos in a procedure modified from that described previously [46]. In short, brains were collected from E18 PS1 transgenic or non-transgenic embryos and kept in ice-cold Hanks’ balanced salt solution (HBSS). The dissected cortices of E18 embryos in a procedure modified from that described previously [46]. In Short, brains were collected from E18 PS1 transgenic or non-transgenic embryos and kept in ice-cold Hanks’ balanced salt solution (HBSS). The dissected cortices were subjected to further analysis. To derive non-neuronal cells (astrocytes, microglia, and oligodendrocytes), dissected cortices from the brains were minced on 35-mm petri dishes on ice. To separate the cells, the minced tissues were suspended in 750 µl 0.25% trypsin/EDTA containing 1 mg/ml DNase I and incubated at 37°C for 15 min. Cells were centrifuged for 5 min at 300×g at room temperature and the cell pellets were dissociated in 5 ml of MEM containing 20% FBS.

Immunoblot and Antibodies

Immediately after euthanasia, mouse brain tissues were dissected and proteins were extracted with TPER buffer (Pierce Chemical Co., Rockford, IL) containing complete protease inhibitor cocktail (Roche, Indianapolis, IN). The protein concentrations in the supernatants were determined by the BCA method (Pierce Chemical Co., Rockford, IL) and equal amount of protein lysates resolved on 4–20% Tris-Glycine SDS PAGE gels, transferred to polyvinylidene difluoride (PVDF) (Invitrogen, Carlsbad, CA) membranes, and probed with following antibodies: anti-Nicastrin [24]; antisera specific for PS1 [47], anti-PEN-2 [48], and anti-Aph1aL antibody (Covance Inc, Princeton, NJ). Blots were probed using monoclonal antibody against actin and rabbit anti-β-tubulinIII antisera (Sigma) were used as loading control. Immunoblots were developed using enhanced chemiluminescence method (Millipore Corp. MA).

In vitro γ-secretase activity assay

The γ-secretase activity of mouse brains was performed as described previously [49]. After mouse brain membranes were extracted in buffer A [50 mM Mes, pH 6.0/5 mM MgCl₂/5 mM CaCl₂/150 mM KCl] with 1×completely protease inhibitor mixture (Boehringer Mannheim), 8 µg of membrane protein was incubated with APP recombinant substrate (1 µM) in the presence of 0.25% CHAPSO in buffer B [50 mM PIPES, pH 7.0/5 mM MgCl₂/5 mM CaCl₂/150 mM KCl] at 37°C. The reactions were stopped by adding RIPA [150 mM NaCl/1.0% NP-40/0.5% sodium deoxycholate/0.1% SDS/30 mM Tris HCl, pH 8.0] and assayed with G2-10 antibody, which recognizes the C-terminus of Aβ40 by ECL.

Filter Trap Assay

Cellulose acetate membranes with 0.2 µm pore size (OE66, Schleicher & Schuell, Keene, NH) were used to trap the aggregates containing Aβ from the brain lysate [50]. Briefly, mouse brains were weighed and then homogenized in 10 volumes of PBS (pH 7.4) containing a protease inhibitor cocktail (Roche, Indianapolis, IN). Homogenates were centrifuged at 3,000 rpm for 5 min at 4°C in a microcentrifuge. The protein concentrations in the supernatants were determined by the BCA method (Pierce Chemical Co., Rockford, IL). Before filtering, the samples were diluted with PBS (100–200 µL) and adjusted to a final concentration of 1% SDS. After filtering, using a 96-well dot-blot apparatus (Bio-Rad Laboratories, Hercules, CA), the membranes were washed twice with 500 µL of PBS (pH 7.4) per well. Proteins trapped by the filter were detected by rabbit anti-Aβ antiserum (Invitrogen, South San Francisco, CA) following conditions used for immunoblotting analysis.

Aβ ELISA Assay

To measure the Aβ levels in vivo, the brains of APPswe;PS1 and APPswe mice were dissected on ice, and homogenized in PBS buffer containing 1% Triton X-100 and complete protease inhibitor
cocktail. After the lysates were centrifuged at 100,000×g for 30 min, the supernatants containing soluble Aβ peptides were collected for assay. The concentrations of total protein extracts were determined by the BCA method (Pierce Chemical Co., Rockford, IL). Aβ40 levels were measured using a quantitative sandwich ELISA kit (Innivetron, South San Francisco, CA) that specifically detects human Aβ.

Histology and Immunohistochemical Analysis

For histological and immunohistochemical analysis of Aβ amyloidosis, mice were perfused with 4% PFA in PBS. Brains were removed, immersion fixed in 4% PFA, and embedded in paraffin, sectioned, and processed. Sections were analyzed immunohistochemically by the peroxidase-antiperoxidase method using antibodies specific for Aβ (6E10(Signet Laboratories, Inc. Dedham, MA) and ubiquitin (Dako Cooperation, Carpinteria, CA).

Measurement of amyloid plaques in mouse brain by unbiased stereology

To assess the Aβ plaque load, one half of the sagittally bisected mouse brain was immersion fixed in 4% PFA and embedded in paraffin. The brains were sectioned at 10 µm and sections were selected at 8-section intervals for analysis using a light microscope interfaced with a Stereo Investigator (MicroBrightfield, Inc.). The quantitative analysis was based on area fraction of ubiquitin immunoreactivity as described previously [51].

Statistical analysis

All data were analyzed statistically by student’s t-test or ANOVA. In all tests, the level of significance was at p<0.05.

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Author Contributions

Conceived and designed the experiments: TL Y-ML SSS DLP PCW. Performed the experiments: TL KA. Analyzed the data: TL Y-ML SSS DLP PCW. Wrote the paper: TL DLP PCW.

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