# γ-Secretase Component Presenilin Is Important for Microglia β-Amyloid Clearance

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**Objective:** The cleavage of amyloid precursor protein by γ-secretase is an important aspect of the pathogenesis of Alzheimer's disease. γ-Secretase also cleaves other membrane proteins (eg, Notch), which control cell development and homeostasis. Presenilin 1 and 2 are considered important determinants of the γ-secretase catalytic site. Our aim was to investigate whether γ-secretase can be important for microglial phagocytosis of Alzheimer's disease β-amyloid.
**Methods:** We investigated the role of γ-secretase in microglia activity toward β-amyloid phagocytosis in cell culture using γ-secretase inhibitors and small hairpin RNA and presenilin-deficient mice.
**Results:** We found that γ-secretase inhibitors impair microglial activity as measured in gene expression, protein levels, and migration ability, which resulted in a reduction of soluble β-amyloid phagocytosis. Moreover, microglia deficient in presenilin 1 and 2 showed impairment in phagocytosis of soluble β-amyloid. Dysfunction in the γ-secretase catalytic site led to an impairment in clearing insoluble β-amyloid from brain sections taken from an Alzheimer's disease mouse model when compared to microglia from wild-type mice.
**Interpretation:** We suggest for the first time, a dual role for γ-secretase in Alzheimer's disease. One role is the cleavage of the amyloid precursor protein for pathologic β-amyloid production and the other is to regulate microglia activity that is important for clearing neurotoxic β-amyloid deposits. Further studies of γ-secretase-mediated cellular pathways in microglia may provide useful insights into the development of Alzheimer's disease and other neurodegenerative diseases, providing future avenues for therapeutic intervention.

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Alzheimer's disease (AD) is the most common type of dementia and affects more than 18 million people worldwide. The main role of the β-amyloid (Aβ) peptide as a mediator in AD is derived from the fact that it accumulates in the brain several decades before the disease is evident.[1] The accumulation of extracellular and intracellular Aβ can adversely affect distinct molecular and cellular pathways, thereby facilitating tau phosphorylation, aggregation, and accumulation of neurofibrillary tangle (NFT) formation.[2] Aβ and NFT exhibit synergistic effects that accelerate neurodegenerative mechanisms involved in metabolism, cellular detoxification, mitochondrial dysfunction, and energy deficiency, resulting in neuritic plaque formation.[3]

Most cases of AD are sporadic and less than 5% are early-onset familial AD (EOFAD) that occur before the age of 65 years.[4] Most genetic cases of AD (75%) relate to the presenilin 1 (PS1)[5] and presenilin 2 (PS2)[6] genes, located on chromosome 14 and 1, respectively. PS1 and PS2 are considered important determinants of the γ-secretase catalytic site that processes the amyloid precursor protein (APP) to Aβ toxic isoforms.[7] It is believed that familial AD mutation leads to a higher production of toxic oligomeric and fibrillar forms of Aβ peptide.[8] One promising therapeutic application is a γ-secretase inhibitor (GSI). In cell cultures and animal models of AD, when GSIs are given, Aβ production and accumulation are significantly reduced.[7] Nevertheless, in rodents, GSIs may cause abnormalities in the gastrointestinal tract, thymus, and spleen.[7] γ-Secretase cleavage activity of type I membrane proteins, including Notch receptors, is important for development and homeostasis.[7] In clinical trials where GSIs were given at mild to

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moderate stages of AD, no clear effect was observed.[9] The activation of microglia cells in the central nervous system (CNS) is the first defense mechanism against the pathologic abnormalities in neurodegenerative diseases.[10] Dysfunction of microglia cells, which alters their sense of the environment, may lead to the development of neurologic diseases such as AD.[11] Mutations that affect activation of microglia in transgenic mouse models of AD promote Aβ deposition and increase mortality in these mice.[10,12] Thus, impairment in microglia cell activity toward degrading Aβ may be a factor in the pathogenesis of AD.[10]

Because presenilin mutations[5,6] accelerate AD pathology, dysfunction of microglia in clearing Aβ may be linked to γ-secretase activity. We found that GSIs impaired microglia activity, as seen in gene expression, and reduced Aβ by phagocytosis. Depletion of the γ-secretase catalytic site of PS1 and PS2 impaired soluble Aβ phagocytosis and clearing of Aβ insoluble plaques.

## Subjects and Methods

### Animals

PS2-deficient ($PS2^{-/-}$) mice of the C57BL/6 background were received from Prof. Bart De Strooper.[13] Mice were housed and maintained in the animal facility of Tel Aviv University, and all experiments complied with protocols approved by the university's animal care committee.

### GSIs

N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; Sigma D5942) or Z-IL-CHO (Calbiochem 565773) was dissolved with 0.1% dimethylsulfoxide (DMSO) and added to cell cultures at final concentrations of 1μM DAPT[14] or 25μM Z-IL-CHO.[15] A structurally related compound, 1μM N-(3,5-difluorophenacetyl)-L-alanine t-butyl ester (DAT),[14] and a cell culture medium with 0.1% DMSO served as control.

### Cell Cultures

We used the N9[16] murine microglia cell line, maintained in a Roswell Park Memorial Institute (RPMI) supplement containing L-Glut (Biological INTD 01-100-1A) with an additional 10% heat-inactivated fetal bovine serum (Biological INTD 04-127-1A) and 1% Pen-Strep (Biological INTD 03-032-1B) in 5% $CO_2$ atmosphere at 37°C.

### Preparations of Microglia Isolated from Primary Mixed Glial Cell Cultures

Primary mixed cultures of wild-type (WT) and $PS2^{-/-}$ mice were prepared from postnatal 1–3-day-old mice and cultured as described.[17]

### Isolation of Adult Microglia

Adult microglia were isolated from 12-week-old WT or $PS2^{-/-}$ mice brains and cultured as described.[18]

### Measurement of Nitrite Levels

Nitric oxide (NO) production was measured in the supernatant using the Quantichrom Nitric Oxide assay kit (DINO-250).

### Evaluation of Aβ (1–42) Phagocytosis by Microglia

N9 cells were incubated with 0.01μg/ml lipopolysaccharide (LPS) for 16 hours, followed by 2-hour incubation with 0.12μM HilyteFlour TM488-Aβ (1–42) (ANASPEC #69479). Microglia were labeled with anti-CD11b antibody (BD Pharmigen 557397) as described[19] and the percentage of Aβ (1–42) phagocytosis was analyzed by fluorescence-activated cell sorting (FACS).

### RNA Analysis

Microglia were analyzed for quantified messenger RNA (mRNA) expression via reverse transcription followed by real-time polymerase chain reaction (RT-PCR) using TaqMan as described and compared with β-actin (ACTB) RNA levels.[19]

### Transfection of Microglia with PS1 Small Hairpin RNA

Transfection of microglia with small hairpin RNA (shRNA) was performed according to the Sigma shRNA protocol (Sigma-Aldrich). Isolation of microglia expressing PS1 shRNA was done with 2μg/ml puromycin. For control we used shRNA against green fluorescent protein. To evaluate the protein level of PS1 (Epitomics 2094-1) in transfected cells we performed western blot analysis as described.[20]

### Intrahippocampal Injections

WT and $PS2^{-/-}$ mice were anesthetized using isoflurane and immobilized in a stereotaxic apparatus. One injection of 0.1μg/ml LPS with 0.12μM HilyteFlour TM488-Aβ (1–42) in a volume of 2μl of phosphate-buffered saline (PBS) was delivered over a 2-minute period into each hippocampus as described.[19] Mice were sacrificed after 2 days for immunohistologic analysis.

### Cell Migration Assay

Cell migration was done as described.[21]

### In Situ Aβ Plaque Clearance

Sagittal brain sections (10μm) were prepared from 9-month-old APP/PS1 mice[22] using a cryostat. Adult microglia ($2 \times 10^5$) were plated on brain sections and incubated at 37°C for 3 days. Quantification of hippocampus and thalamus Aβ burden was done as described.[23] Staining was performed on 5 consecutive sections per animal and repeated 4 times per group in a blinded fashion using Imaging Research software from the National Institutes of Health in an unbiased stereological approach.

### In Vivo Aβ (1–42) Phagocytosis by Peritoneal Macrophage

Calcium-free and magnesium-free PBS (0.5ml) was injected intraperitoneally into 12-week-old C57BL/6 male mice overnight

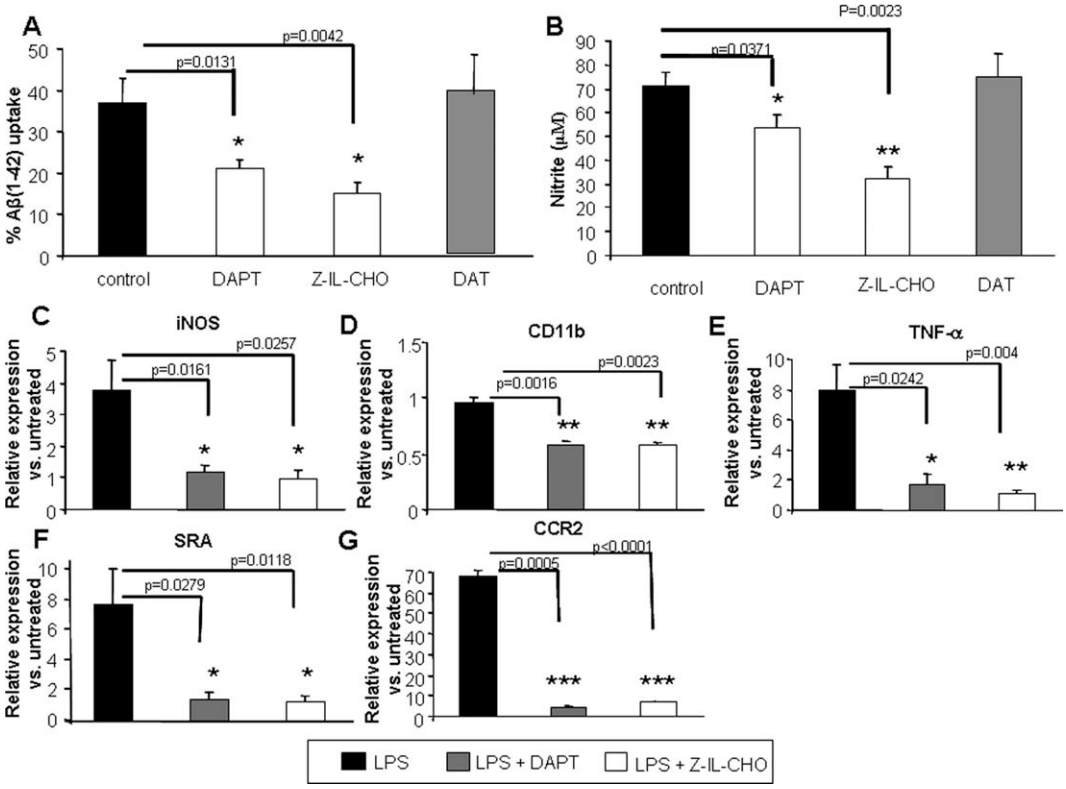


**FIGURE 1: GSIs reduce phagocytosis of A$\beta$ by N9 microglia cell line. (A) Incubation with GSIs significantly reduces phagocytosis of A$\beta$ by N9 cells, as measured by FACS: 1$\mu$M DAPT (n = 6) and 25$\mu$M Z-IL-CHO (n = 6) vs control (co-stimulation of cells with 0.01$\mu$g/ml LPS and 0.12$\mu$M A$\beta$). (B) Incubation with GSIs reduces nitrite levels in cell culture supernatant 1$\mu$M DAPT (n = 8) and 25$\mu$M Z-IL-CHO (n = 8). (C) Analysis of gene expression iNOS, (D) CD11b, (E) TNF-$\alpha$, (F) SRA, and (G) CCR2 in N9 microglia following incubation with and without GSIs. Results are presented as relative expression of the gene of interest vs mouse ACTB RNA levels and compared to untreated (n = 4). Results are presented as mean ± SEM. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$.**

(n = 8). Then, 0.5$\mu$g HilyteFlour TM488-A$\beta$ (1–42) in 0.5ml PBS was injected intraperitoneally for 2 hours. Mice were euthanized with $CO_2$ and peritoneal macrophages were collected with 10ml ice-cold PBS. FACS analysis was used to determine the percent of A$\beta$ uptake by macrophages ($CD11b^+$).

### *Statistical Analysis*

Data were compared using either a 2-tailed Student *t* test when 2 groups were compared or with 1-way analysis of variance analysis (Bonferroni test) when 3 or more groups were analyzed. Statistical significance was considered at $p < 0.05$.

## Results

### *GSIs Reduce Phagocytosis of Aβ by Microglia*

We first investigated whether treatment with DAPT[14] and Z-IL-CHO[15] could affect phagocytosis of A$\beta$ by microglia compared to DAT.[14] As shown in Figure 1A and Supporting Figure S1A, phagocytosis of A$\beta$ (1–42) was significantly reduced, 43% with DAPT and 59.5% with Z-IL-CHO compared to nontreated cells or DAT-treated cells used as controls. Although this effect was magnified in the presence of LPS, it was not LPS-dependent because GSIs also reduced phagocytosis of A$\beta$ (1–42) when microglia were incubated without LPS (Supporting Fig S2). GSIs also reduced phagocytosis of fluorescent latex beads by N9 cells with and without LPS incubation (Supporting Fig S3). We repeated FACS analysis of fluorescent A$\beta$ uptake by N9 cells following incubation with trypan blue that quenched the extracellular fluorescence[24] and had similar results as when quenching was absent, suggesting that the fluorescent A$\beta$ was being phagocytosed to the microglia cell. We believe that $\gamma$-secretase is required for microglia phagocytosis activity.

An elevated level of NO is a marker of microglia activity and may be operative in neurodegenerative diseases such as AD.[25] Because NO may be in different oxidative stages, we oxidized the cell supernatant to nitrite to provide a better indication of total NO production by microglia. As shown in Figure 1B, following co-stimulation with 0.01$\mu$g/ml LPS and A$\beta$ (1–42), nitrite levels were significantly reduced, 25% with DAPT and 54% with Z-IL-CHO. No significant effect was obtained using DAT.

To investigate microglia gene expression, we used quantitative RT-PCR measurements of microglia following co-stimulation with LPS and A$\beta$ (1–42) (as control) or

with GSIs. We found (see Fig 1C) that in correlation with the reduction of NO levels in the presence of GSIs toward soluble Aβ (1–42), a reduction occurred in the inducible nitric oxide synthase (iNOS)-relative expression vs untreated cells: 68% with DAPT and 74% with Z-IL-CHO vs control. CD11b is a marker for MAC-1 integrin that plays a role in migration of microglia. CD11b expression (see Fig 1D) was reduced in the presence of GSIs as measured by relative expression vs untreated cells: 40% with DAPT and 40% with Z-IL-CHO vs control.

As shown in Figure 1E, the expression of microglia activation marker, tumor necrosis factor-α (TNF-α), was significantly reduced in the presence of GSIs: 80% reduction with DAPT and 86% reduction with Z-IL-CHO vs control. Previous reports indicated the elevation of scavenger receptor A (SRA)[26] in mediating phagocytosis of Aβ by microglia. We found (see Fig 1F) a significant reduction of 80% in the expression of SRA in microglia in the presence of DAPT and Z-IL-CHO vs control.

C-C chemokine receptor 2 (CCR2) is the main receptor for C-C chemokine ligand 2 (CCL2), a major chemotactic peptide for microglia and monocytes. In $CCR2^{-/-}$ mice, microglia fail to migrate toward Aβ.[12] We found (see Fig 1G) a dramatic reduction of 90% in expression of CCR2 in microglia in the presence of DAPT and Z-IL-CHO vs control.

The inhibitory effects of GSIs on microglia activity were also observed in protein levels shown by enzyme-linked immunosorbent assay, FACS, and Western blotting (Supplementary Fig S4). No evidence for toxicity was apparent after incubation with GSIs as measured by methylthiotetrazole and by levels of control mRNA gene (ACTB) (Supporting Fig S5), suggesting that a reduction in microglia activity relates to an impairment in γ-secretase activity.

### *PS2 Deficiency Leads to Impairment in the Phagocytosis of Aβ by Primary Microglia*

Because PS2 is an important component in the γ-secretase active site, we compared $PS2^{-/-}$ to WT microglia. $PS2^{-/-}$ mice are viable and fertile and develop only mild pulmonary fibrosis.[13] Although mixed glial cell cultures include astrocytes and oligodendrocytes, perhaps affecting microglia activity, mixed cultures better mimic the physiologic environment than isolated microglia cultures. In both mixed glial cultures of $PS2^{-/-}$ and WT, the percentage of microglia ($CD11b^+$) was 12% as indicated by FACS analysis, demonstrating that $PS2^{-/-}$ does not affect the number of microglial cells in the brain (Supporting Fig S6). We used FACS to determine the percentage of Aβ (1–42) phagocytosis by microglia ($CD11b^+$). As shown in Figure 2A and Supporting Figure S1B, WT microglia ($CD11b^+$) that phagocytosed soluble Aβ were reduced 50% compared to microglia from $PS2^{-/-}$ animals. To evaluate γ-secretase activity, we repeated the experiment in the presence of Z-IL-CHO. We found (see Fig 2A) reductions of 58% and 39% in $CD11b^+$Aβ (1–42)+ from WT and $PS2^{-/-}$, respectively. These results suggest that PS2 is important for γ-secretase activity; however, other essential components of γ-secretase, such as PS1, may also facilitate its activity.

To further assess microglia activity in $PS2^{-/-}$ vs WT mice, we measured the percent of fold decrease in nitrite following stimulation in the presence of GSIs. Figure 2B shows the significant reduction of nitrite secretion in $PS2^{-/-}$ vs WT, which demonstrated less activation of $PS2^{-/-}$ microglia. Incubation in the presence of GSIs resulted in a 63% reduction of nitrite in WT microglia and no significant reduction in $PS2^{-/-}$ microglia.

Using FACS, we isolated the microglia ($CD11b^+$) population that phagocytosed fluorescent Aβ and investigated the gene expression profile as relative expression vs untreated cells. As shown in Figure 2C, gene expression of activation markers was significantly different between WT vs $PS2^{-/-}$, with 91% and 47% reductions, respectively, in interleukin 6 (IL-6) and TNF-α (see Fig 2D). Incubation with a GSI resulted in an 86% reduction in the expression of IL-6 in WT and a 54% reduction in $PS2^{-/-}$ microglia. We found a 55% reduction in the expression of TNF-α in WT microglia but no significant reduction in TNF-α expression in $PS2^{-/-}$ microglia.

As shown in Figure 2E and F, no significant difference was found between WT and $PS2^{-/-}$ microglia in CD11b and SRA gene expression. These results might be related to the uniqueness of the sorted population, $CD11b^+A\beta^+$ in both cases. However, we found a significant reduction in the presence of GSI, in both WT and $PS2^{-/-}$ microglia.

A 92% and 59% reduction of CD11b gene expression was found in WT microglia and $PS2^{-/-}$ microglia, respectively. In the presence of a GSI, we found (see Fig 2F) a 90% reduction in SRA gene expression in WT microglia but only a 68% reduction in $PS2^{-/-}$ microglia. Levels of control mRNA genes remained unchanged in microglia from WT and $PS2^{-/-}$ mice, suggesting that differences between WT and $PS2^{-/-}$ relate to the activation status of microglia. Similar results were obtained in microglia activation from $PS2^{-/-}$ vs WT mice without LPS incubation (Supporting Fig S7).

### *PS1 Silencing Reduces Phagocytosis of Aβ*

To investigate the effects of PS1 depletion on microglia cells, we used the shRNA approach because the PS1 knockout gene is lethal in mice. We received 5 different

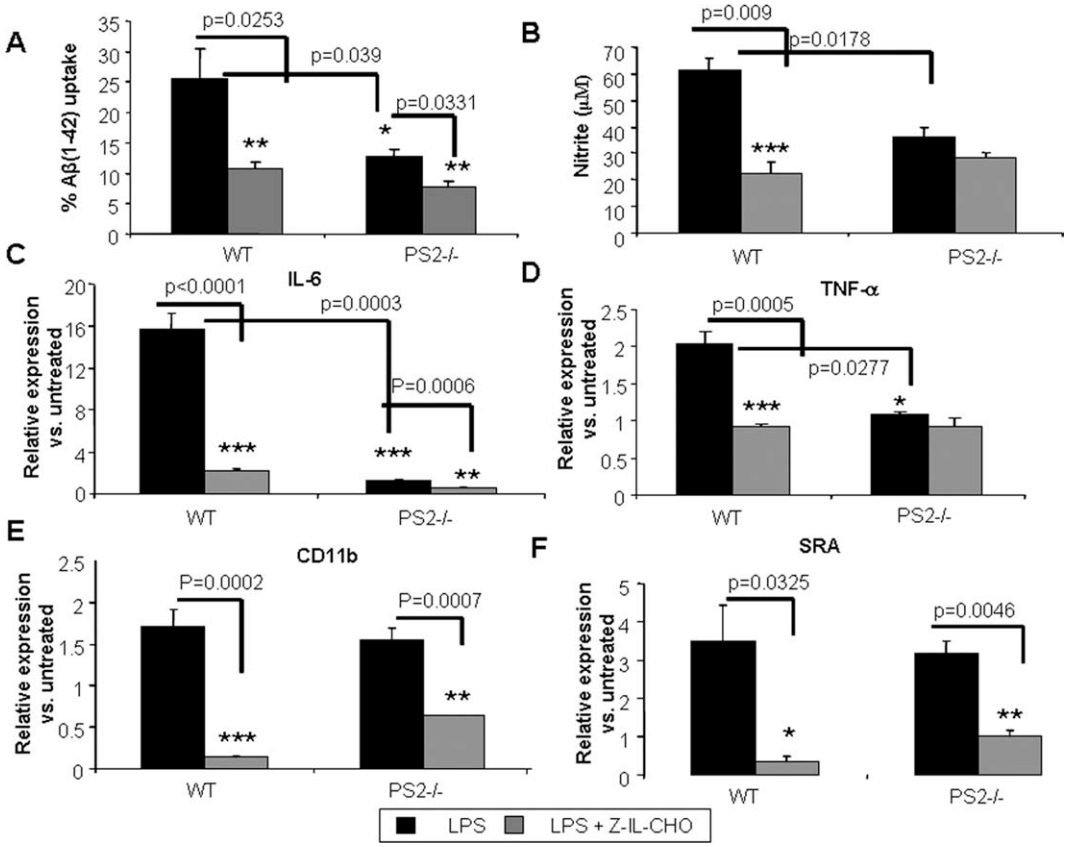


**FIGURE 2: PS2 deficiency leads to impairment in phagocytosis of soluble A$\beta$ by microglia. Primary glial mixed cultured cells were activated by 0.1$\mu$g/ml LPS with and without GSI (25$\mu$M Z-IL-CHO) overnight. Following overnight activation with A$\beta$, FITC was added to cells for 2 hours. Results are presented as mean ± SEM. (A) FACS analysis of phagocytosis of fluorescent A$\beta$ by WT vs PS2$^{-/-}$ microglia (CD11b$^{+}$) (n = 4). (B) Nitrite ($\mu$M) levels as measured from primary glial cell culture supernatant of WT vs PS2$^{-/-}$ mixed glial cell culture (n = 5). (C) Analysis of gene expression IL-6, (D) TNF-$\alpha$, (E) CD11b, and (F) SRA of WT vs PS2$^{-/-}$ microglia (n = 4). Results are presented as relative expression vs untreated of the gene of interest and compared to mouse ACTB RNA levels. Results are presented as mean ± SEM. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$. FITC = fluorescein isothiocyanate.**

shRNA vectors (Sigma) targeted against PS1. To test whether the gene was silenced, western blotting determined that transfected N9 cells demonstrated a 33% silencing (Fig 3A, B). As shown in Figure 3C and Supporting Figure S1C, PS1 silencing reduced microglia phagocytosis of soluble A$\beta$ (1–42) by 33.1% vs control. To evaluate $\gamma$-secretase activity in shRNA PS1-transfected microglia, we repeated the experiment in the presence of DAPT. We found (see Fig 3C) a further 16.7% reduction of CD11b$^{+}$A$\beta$ $^{+}$ from shRNA PS1-transfected microglia. These results suggest that although PS1 is important for $\gamma$-secretase activity, other components such as PS2 that facilitate this activity are also important.

### *A GSI or Dysfunction in the Catalytic Site Impairs Microglia Migration*

Microglia were tested for their ability to migrate toward CCL2, in the presence and absence of GSIs. As shown in Figure 4A and Supporting Figure S8A, both GSIs significantly reduced the migration of N9 cells, 67.7% in DAPT and 68.2% in Z-IL-CHO. As shown in Figure 4B and C and Supporting Figure S8B and C, the migration ability of PS2$^{-/-}$ microglia and microglia transfected with PS1 shRNA was reduced by 62.44% and 48.3%, respectively.

### *PS2 Deficiency Reduces Microglia Activation and Aβ Clearance In Vivo*

To test the effect of PS2 on the ability of microglia cells to phagocytize soluble A$\beta$ (1–42) in vivo, fluorescent A$\beta$ (1–42) was injected into the hippocampus of WT and PS2$^{-/-}$ mice. After 48 hours, a significant increase was seen in microglia activation in WT mice compared to PS2$^{-/-}$ mice (Fig 5). A 40% reduction in the clearance of A$\beta$ in PS2$^{-/-}$ mice compared to WT mice was also seen.

### *Adult Mouse Microglia Clear Insoluble Aβ Plaques in an In Situ Assay*

To determine the difference between WT and PS2$^{-/-}$ adult microglia and their response to brain A$\beta$ deposits,

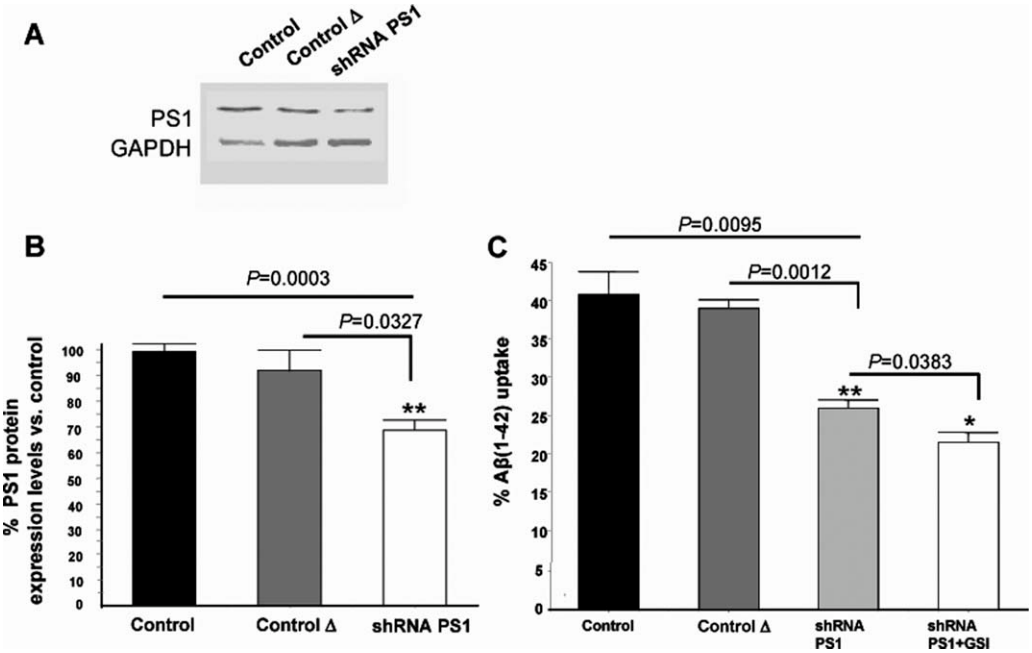


**FIGURE 3: PS1 silencing in microglia leads to a reduction of phagocytosis of soluble A$\beta$. (A) Western blot representative figures and (B) analysis of PS1 expression in N9 microglia cell line transfected with shRNA against PS1. Control group is cells transfected with control shRNA (shRNA against green fluorescent protein) and control Δ is an internal control cells transfected by shRNA against PS1 but that showed no reduction in protein expression (n = 5). (C) FACS analysis of phagocytosis of soluble A$\beta$ following transfection with shRNA against PS1 and co-incubation with GSI (n = 5). Results are presented as mean ± SEM. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$.**

we isolated adult microglia as described.[23] We plated adult microglia from WT or PS2$^{-/-}$ on unfixed insoluble APP-rich brain sections from transgenic mice expressing human APP[22] (Fig 6A). We discovered that microglia from WT mice reduced 43% of insoluble A$\beta$ in the hippocampus brain region compared to the untreated section, whereas microglia from PS2$^{-/-}$ mice reduced only 17% of insoluble A$\beta$ (see Fig 6B and C). We obtained similar results for other brain regions, such as the lateral posterior thalamic nucleus; WT microglia cleared 56% and PS2$^{-/-}$ microglia cleared only 8% of insoluble A$\beta$.

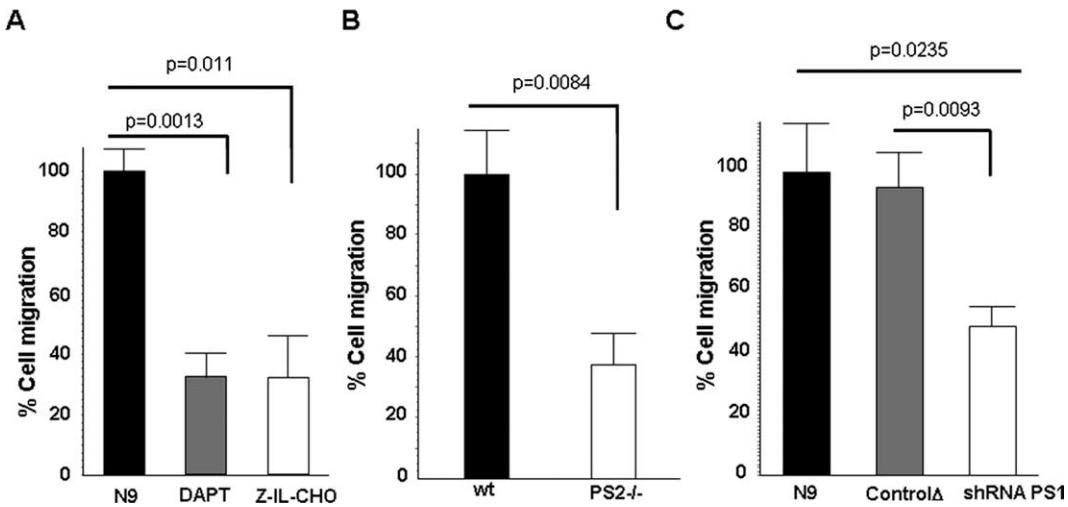


**FIGURE 4: γ-Secretase dysfunction impairs microglia migration ability. Migration was measured by the number of cells that migrated through an 8-$\mu$m membrane toward medium containing 20% serum and 50ng/ml CCL2. (A) Migration of N9 cell microglia was measured following incubation with GSI (n = 5). (B) Migration of microglia isolated from primary mixed glial cultured cells from WT mice vs PS2$^{-/-}$ mice (n = 7). (C) Migration of microglia cells from N9 transfected cells with shRNA against PS1 vs control Δ and control cells. Results are presented as mean ± SEM. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$.**

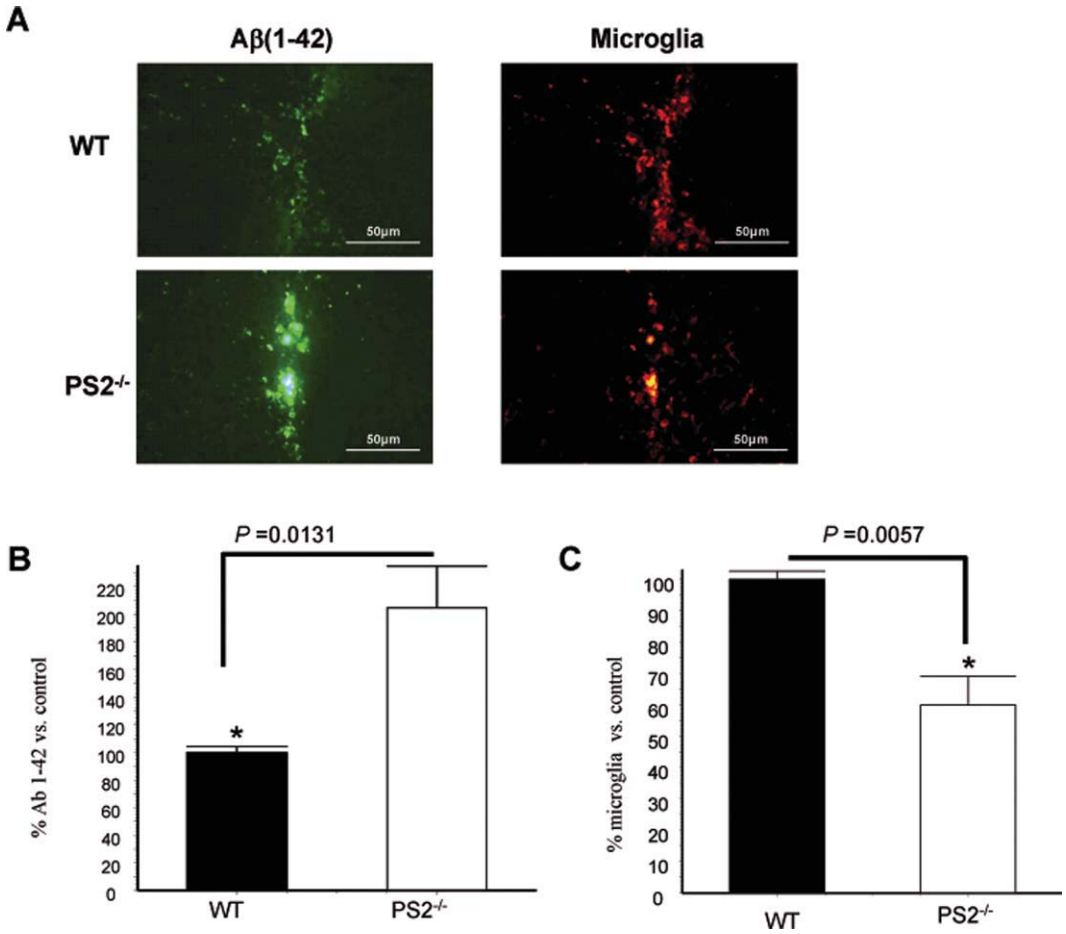


**FIGURE 5: PS2 deficiency reduces microglia activation as well as A$\beta$ clearance in vivo. WT and PS2$^{-/-}$ mice were anesthetized and immobilized in a stereotaxic apparatus. (A) LPS, 0.1$\mu$g/ml, with 0.12$\mu$M fluorescent A$\beta$ was injected into each hippocampus (n = 3) and immunostaining was preformed for A$\beta$ (fluorescent) and microglia (Iba1) (original magnification ×20). Quantification of (B) hippocampus A$\beta$ and (C) microglia (Iba1$^{+}$) was done by calculating the percentage of the stained area from the total hippocampal region in PS2 vs WT brains. PS2 mice exhibited reduced microglia activation as well as reduced clearance of A$\beta$. Results are presented as mean ± SEM. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]**

To investigate the profile gene expression in adult microglia, we performed RT-PCR (Fig 7). We focused on 3 different gene expression profiles linked to microglia function toward A$\beta$: activation markers (CD11b, iNOS, TNF-$\alpha$, IL-6, SRA, and Toll-like receptor 2 [TLR-2])[10]; migration marker (CCR2)[12]; and expression of enzymes that degrade A$\beta$ such as insulin-degrading enzyme (IDE)[27] and matrix metalloproteinase-9 (MMP-9).[28] In PS2$^{-/-}$ microglia compared to WT microglia, significant downregulation in activation markers occurred: reductions of 53% in CD11b, 81% in iNOS, 39% in TNF-$\alpha$, 36% in IL-6, 50% in SRA, and 40% in TLR-2. A 48% reduction occurred in expression of CCR2, a crucial cell migration marker, in PS2$^{-/-}$ vs WT. Microglia reportedly express A$\beta$-degrading enzymes[29,30]; therefore, we investigated whether PS2 deficiency can influence the expression of those genes. We obtained reductions of 45% in IDE and 63% in MMP-9 in adult microglia from PS2$^{-/-}$ vs WT mice.

### *PS2 is Important for Phagocytosis of Soluble A$\beta$ by Peritoneal Macrophages In Vivo*

Peripheral monocyte migration to the CNS was considered important for clearance of amyloid plaques.[31] We investigated differences between WT and PS2$^{-/-}$ macrophage activity toward soluble A$\beta$. We investigated the in vivo uptake of soluble A$\beta$ (1–42) by peritoneal macrophages.[32] Although in our experimental design (Fig 8A) other cell types may affect phagocytosis of A$\beta$ by macrophages, our paradigm more closely mimics the true physiologic environment as opposed to an experiment that includes only isolated peritoneal macrophages. We found (see Fig 8B and Supporting Fig 1D) a 48% reduction in phagocytosis of A$\beta$ in PS2$^{-/-}$ vs WT peritoneal macrophages. We found a 64% reduction in CCR2 and a 65% reduction in IDE in PS2$^{-/-}$ macrophages vs WT (see Fig 8C, D). These results demonstrate that $\gamma$-secretase is essential for macrophage migration and A$\beta$ phagocytosis.

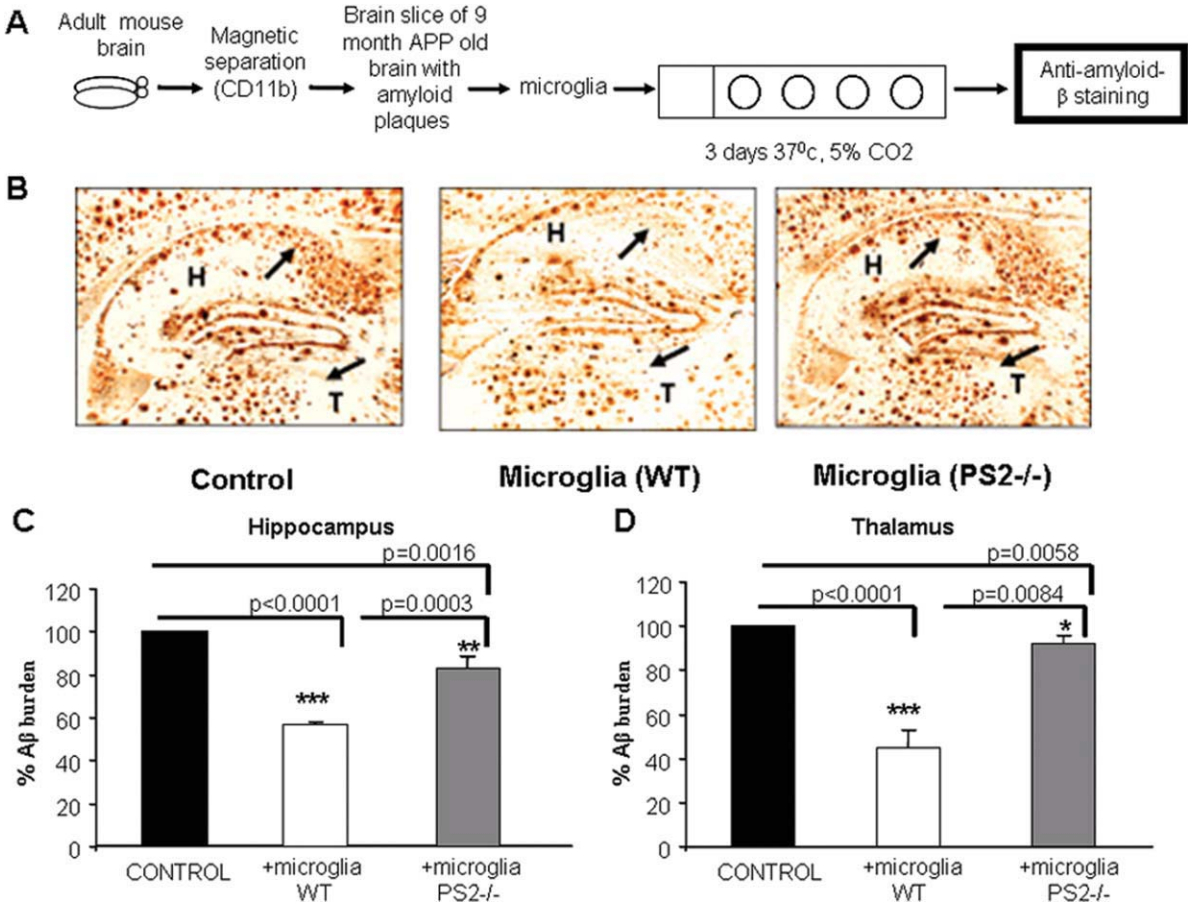


**FIGURE 6: Adult mouse microglia clear insoluble Aβ plaques in an in situ assay. Adult mouse microglia were plated on frozen brain sections from a mouse model of AD for 72 h. Aβ level was measured by staining with anti-Aβ antibodies. (A) Scheme of in situ assay of insoluble Aβ degradation by adult microglia. (B) Adult mouse microglia from WT mouse show increased degradation of hippocampal insoluble Aβ plaques in the in situ assay as compared to microglia from a PS2$^{-/-}$ mouse (n = 7) (original magnification ×10). Statistical analysis of Aβ degradation by microglia from (C) hippocampus and (D) lateral posterior thalamic region (LPL) (n = 7). Results are presented as mean ± SEM. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]**

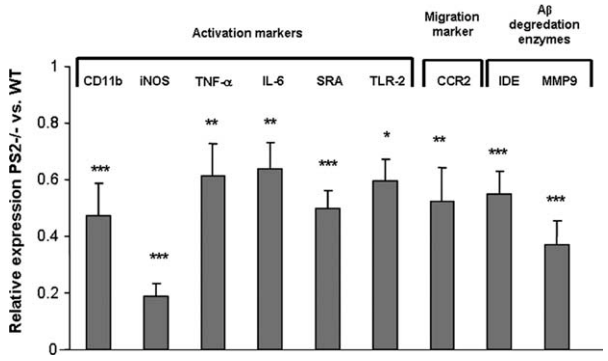


**FIGURE 7: γ-Secretase dysfunction reduces adult microglia gene expression. Real-time PCR analysis of adult microglia from WT vs PS2$^{-/-}$ mice. Relative expression analysis of the gene of interest (CD11b, iNOS, TNF-α, TLR2, SRA, CCR2, IDE, and MMP-9) from PS2$^{-/-}$ microglia vs WT adult microglia and compared to mouse ACTB RNA levels (n = 4). Results are presented as mean ± SEM. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$.**

## Discussion

We have demonstrated for the first time a dual role for γ-secretase in the pathogenesis of AD. Although a previous publication[7] showed the importance of γ-secretase in the formation of neurotoxic Aβ, we demonstrated the role of γ-secretase in mediating microglia activity in phagocytosis and clearing Aβ from the brain.

Activated microglia represent the major source of brain inflammatory factors in neurodegenerative diseases such as AD.[11] In addition, microglia have the ability to clear and dispose of foreign materials in the brain, such as toxic Aβ. Human brains containing high amyloid loads demonstrate a significantly higher degree of microglia dystrophy than nondemented, amyloid-free control brains.[33] This suggests that the degeneration of microglia is a factor in the pathogenesis of AD. We hypothesize that a dysfunction of microglia may be linked to γ-secretase activity in the same way presenilin mutations accelerate AD pathology.

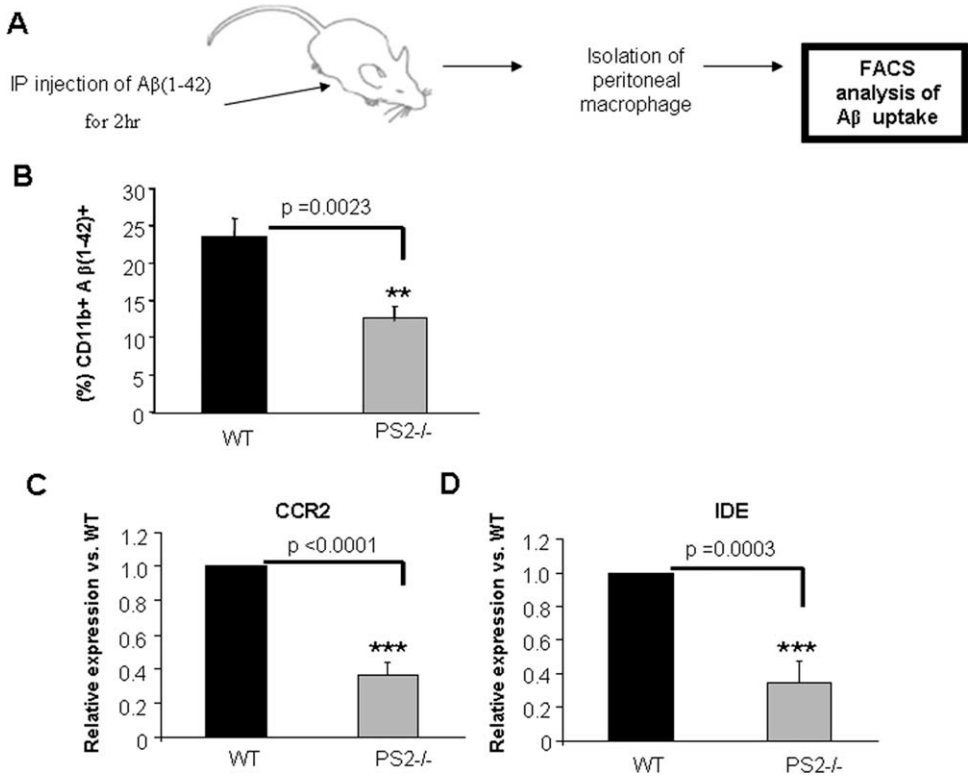


**FIGURE 8: PS2 is essential for activation of peritoneal macrophages to phagocytize A$\beta$ in vivo. (A) Scheme of in vivo assay of soluble A$\beta$ phagocytosis by peritoneal macrophages. (B) Macrophages from PS2$^{-/-}$ mice (n = 9) show significant reduction in uptake of A$\beta$ vs macrophages from WT mice (n = 9) as measured by FACS. (C) Relative expression analysis of the gene of interest, CCR2, and (D) IDE from PS2$^{-/-}$ macrophages vs WT macrophages and compared to mouse ACTB RNA levels. Results are presented as mean ± SEM. **$p < 0.005$; ***$p < 0.0005$.**

To assess this, we used 2 types of GSIs: DAPT, a transition state analog inhibitor,[14] and Z-IL-CHO, a small peptide-based inhibitor.[15] GSIs impair microglia activity, as indicated by gene expression. This impaired activity leads to a reduction of A$\beta$ phagocytosis. GSIs lead to a reduction in $\gamma$-secretase activity and may also affect other intramembrane aspartyl proteases, such as signal peptide peptidase (SPP), which may be also involved in microglia activity.

As part of the regulated intramembrane proteolysis process, $\gamma$-secretase cleaves, besides APP, other type 1 transmembrane proteins such as Notch and CD44, which modulate microglia activity.[34,35] In line with our results, emerging evidence suggests that inhibition of $\gamma$-secretase activity may interfere with the immune system.[36] The ability of GSIs to modulate Notch signaling may prove beneficial for treating other clinical conditions such as acute multiple sclerosis[37] and ischemic stroke.[38] These results suggest that GSIs have a role in reducing proinflammatory T cells. Studies by Weggen and colleagues[39] demonstrate that a subset of nonsteroidal anti-inflammatory drugs (NSAIDs) can modulate $\gamma$-secretase, leading to a change in the A$\beta$40/42 ratio. In addition, some NSAIDs (eg, ibuprofen) decrease the numbers of microglia activations in an AD mouse model.[40]

PS1 and PS2 are part of the $\gamma$-secretase catalytic site. Adult microglia isolated from animals bearing a specific depletion in the $\gamma$-secretase catalytic site (eg, PS2$^{-/-}$ mice) have a significantly reduced ability to clear soluble A$\beta$ in vivo and insoluble A$\beta$ plaques in situ. Interestingly, although no evidence was found for impairment in early development of microglia from PS2$^{-/-}$ primary mixed glial cultures, microglia activity was significantly reduced. This suggests that PS2 is involved in the activation of microglia from the surveying stage. Consistent with this phenomenon, Behbahani and colleagues[41] showed that PS2 plays a role in mitochondrial membrane potential activity and cell calcium levels that may result in activity impairment. PS1 associates with microtubules and microfilaments and PS1 dysfunction may reduce cytoskeletal association.[42] Therefore, PS dysfunction may result in changes in microglia cytoskeleton leading to alterations in microglia endocytosis of A$\beta$.

We found a significant reduction in gene expression, protein levels, and migration ability, both from the use of GSIs and a specific impairment in the $\gamma$-secretase catalytic site (PS1 or PS2).

In EOFAD, the genetics of PS1 and PS2 are linked to point mutations and not depletion.[4] Nevertheless, the cross between APP mice with PS1[43] or

PS2[44] bearing human-specific point mutations resulted in accelerated amyloid deposition. This may also be linked to a deficiency in Aβ phagocytosis given the role of microglia in the clearance of amyloid.[11] Furthermore, Lee and colleagues[45] found that the effect of overexpression of human PS1 mutations in microglia cells results in a proinflammatory response. A recent report suggests that the Notch pathway plays an important role in controlling inflammatory reactions in the CNS.[34] Previous studies[46,47] suggested that proinflammatory microglia are impaired during phagocytosis activity, possibly linked to an impairment in the γ-secretase catalytic site.[48] Microglia ability to phagocytize Aβ was previously linked to expression of scavenger receptors such as SRA.[26] Therefore, alteration in γ-secretase activity reduce SRAs, which may then reduce microglia Aβ phagocytosis.

Microglia have many similarities to macrophages and are thought to originate from the same lineage. A recent report[31] suggests that blood-derived microglia have a greater ability than their resident counterparts to eliminate amyloid deposits by cell-specific phagocytic mechanisms. We found that PS2 deficiency leads to the impairment of peritoneal macrophage migration and phagocytosis of Aβ in vivo.

We suggest for the first time a dual role of γ-secretase in AD. The cleavage of APP by γ-secretase is important in the pathogenesis of AD and amyloid accumulation. Thus, γ-secretase is a target for development of AD therapies and GSIs. As we demonstrated, γ-secretase mediates microglia activity. A mutation in presenilin that affects γ-secretase activity may result in microglia dysfunction and enhance amyloid accumulation. Because GSIs may impair microglia activity, the next step is to design unique GSIs that specifically reduce APP cleavage yet minimize their effect on receptors that might affect microglia phagocytosis activity.

Microglia cells do not have uniform properties and different microglia phenotypes probably have the potential to be beneficial or harmful in AD. The investigation of γ-secretase-mediated cellular pathways in microglia may provide useful therapeutic intervention targets for neurodegenerative diseases such as AD.

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## Potential Conflicts of Interest

Nothing to report.

## References

1. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 2002;297:353–356.
2. Lee VM, Trojanowski JQ. Progress from Alzheimer's tangles to pathological tau points towards more effective therapies now. J Alzheimers Dis 2006;9:257–262.
3. Aliev G, Seyidova D, Lamb BT, et al. Mitochondria and vascular lesions as a central target for the development of Alzheimer's disease and Alzheimer disease-like pathology in transgenic mice. Neurol Res 2003;25:665–674.
4. Sorbi S, Forleo P, Tedde A, et al. Genetic risk factors in familial Alzheimer's disease. Mech Ageing Dev 2001;122:1951–1960.
5. Goldman JS, Reed B, Gearhart R, et al. Very early-onset familial Alzheimer's disease: a novel presenilin 1 mutation. Int J Geriatr Psychiatry 2002;17:649–651.
6. Levy-Lahad E, Wasco W, Poorkaj P, et al. Candidate gene for the chromosome 1 familial Alzheimer's disease locus. Science 1995; 269:973–977.
7. Wolfe MS. Gamma-secretase: structure, function, and modulation for Alzheimer's disease. Curr Top Med Chem 2008;8:2–8.
8. Haass C, Selkoe DJ. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Nat Rev Mol Cell Biol 2007;8:101–112.
9. Imbimbo BP. Therapeutic potential of gamma-secretase inhibitors and modulators. Curr Top Med Chem 2008;8:54–61.
10. Farfara D, Lifshitz V, Frenkel D. Neuroprotective and neurotoxic properties of glial cells in the pathogenesis of Alzheimer's disease. J Cell Mol Med 2008;12:762–780.
11. Streit WJ. Microglia and Alzheimer's disease pathogenesis. J Neurosci Res 2004;77:1–8.
12. El Khoury J, Toft M, Hickman SE, et al. Ccr2 deficiency impairs microglial accumulation and accelerates progression of Alzheimer-like disease. Nat Med 2007;13:432–438.
13. Herreman A, Hartmann D, Annaert W, et al. Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. Proc Natl Acad Sci U S A 1999;96:11872–11877.
14. Micchelli CA, Esler WP, Kimberly WT, et al. Gamma-secretase/presenilin inhibitors for Alzheimer's disease phenocopy Notch mutations in Drosophila. FASEB J 2003;17:79–81.
15. McLendon C, Xin T, Ziani-Cherif C, et al. Cell-free assays for gamma-secretase activity. FASEB J.2000;14:2383–2386.
16. Mayo L, Stein R. Characterization of LPS and interferon-gamma triggered activation-induced cell death in N9 and primary microglial cells: induction of the mitochondrial gateway by nitric oxide. Cell Death Differ 2007;14:183–186.
17. Saura J, Tusell JM, Serratosa J. High-yield isolation of murine microglia by mild trypsinization. Glia 2003;44:183–189.
18. Hickman SE, Allison EK, El Khoury J. Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. J Neurosci 2008;28:8354–8360.
19. Frenkel D, Puckett L, Petrovic S, et al. A nasal proteosome adjuvant activates microglia and prevents amyloid deposition. Ann Neurol 2008;63:591–601.
20. Kimberly WT, Xia W, Rahmati T, et al. The transmembrane aspartates in presenilin 1 and 2 are obligatory for gamma-secretase

activity and amyloid beta-protein generation. J Biol Chem 2000; 275:3173–3178.

21. Chan A, Hummel V, Weilbach FX, et al. Phagocytosis of apoptotic inflammatory cells downregulates microglial chemoattractive function and migration of encephalitogenic T cells. J Neurosci Res 2006;84:1217–1224.
22. Oakley H, Cole SL, Logan S, et al. Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. J Neurosci 2006;26: 10129–10140.
23. Wyss-Coray T, Loike JD, Brionne TC, et al. Adult mouse astrocytes degrade amyloid-beta in vitro and in situ. Nat Med 2003;9: 453–457.
24. Li H, Black NP, Chokshi A, et al. High-throughput screening for fatty acid uptake inhibitors in humanized yeast identifies atypical antipsychotic drugs that cause dyslipidemias. J Lipid Res 2008;49: 230–244.
25. Lee SC, Zhao ML, Hirano A, Dickson DW. Inducible nitric oxide synthase immunoreactivity in the Alzheimer disease hippocampus: association with Hirano bodies, neurofibrillary tangles, and senile plaques. J Neuropathol Exp Neurol 1999;58:1163–1169.
26. El Khoury J, Hickman SE, Thomas CA, et al. Scavenger receptor-mediated adhesion of microglia to beta-amyloid fibrils. Nature 1996;382:716–719.
27. Farris W, Mansourian S, Chang Y, et al. Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo. Proc Natl Acad Sci U S A 2003;100:4162–4167.
28. Yan P, Hu X, Song H, et al. Matrix metalloproteinase-9 degrades amyloid-beta fibrils in vitro and compact plaques in situ. J Biol Chem 2006;281:24566–24574.
29. Qiu WQ, Walsh DM, Ye Z, et al. Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation. J Biol Chem 1998;273:32730–32738.
30. Qiu WQ, Ye Z, Kholodenko D, et al. Degradation of amyloid beta-protein by a metalloprotease secreted by microglia and other neural and non-neural cells. J Biol Chem 1997;272: 6641–6646.
31. Simard AR, Soulet D, Gowing G, et al. Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. Neuron 2006;49:489–502.
32. Wan L, Pooyan S, Hu P, et al. Peritoneal macrophage uptake, pharmacokinetics and biodistribution of macrophage-targeted PEG-fMLF (N-formyl-methionyl-leucyl-phenylalanine) nanocarriers for improving HIV drug delivery. Pharm Res 2007;24:2110–2119.
33. Flanary BE, Sammons NW, Nguyen C, et al. Evidence that aging and amyloid promote microglial cell senescence. Rejuvenation Res 2007;10:61–74.
34. Grandbarbe L, Michelucci A, Heurtaux T, et al. Notch signaling modulates the activation of microglial cells. Glia 2007;55: 1519–1530.
35. Rolls A, Shechter R, London A, et al. Two faces of chondroitin sulfate proteoglycan in spinal cord repair: a role in microglia/macrophage activation. PLoS Med 2008;5:e171.
36. Maillard I, Adler SH, Pear WS. Notch and the immune system. Immunity 2003;19:781–791.
37. Minter LM, Turley DM, Das P, et al. Inhibitors of gamma-secretase block in vivo and in vitro T helper type 1 polarization by preventing Notch upregulation of Tbx21. Nat Immunol 2005;6:680–688.
38. Arumugam TV, Chan SL, Jo DG, et al. Gamma secretase-mediated Notch signaling worsens brain damage and functional outcome in ischemic stroke. Nat Med 2006;12:621–623.
39. Weggen S, Eriksen JL, Das P, et al. A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity. Nature 2001;414:212–216.
40. Lim GP, Yang F, Chu T, et al. Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. J Neurosci 2000;20:5709–5714.
41. Behbahani H, Shabalina IG, Wiehager B, et al. Differential role of presenilin-1 and -2 on mitochondrial membrane potential and oxygen consumption in mouse embryonic fibroblasts. J Neurosci Res 2006;84:891–902.
42. Pigino G, Pelsman A, Mori H, Busciglio J. Presenilin-1 mutations reduce cytoskeletal association, deregulate neurite growth, and potentiate neuronal dystrophy and tau phosphorylation. J Neurosci 2001;21:834–842.
43. Trinchese F, Liu S, Battaglia F, et al. Progressive age-related development of Alzheimer-like pathology in APP/PS1 mice. Ann Neurol 2004;55:801–814.
44. Richards JG, Higgins GA, Ouagazzal AM, et al. PS2APP transgenic mice, coexpressing hPS2mut and hAPPswe, show age-related cognitive deficits associated with discrete brain amyloid deposition and inflammation. J Neurosci 2003;23:8989–9003.
45. Lee J, Chan SL, Mattson MP. Adverse effect of a presenilin-1 mutation in microglia results in enhanced nitric oxide and inflammatory cytokine responses to immune challenge in the brain. Neuromolecular Med 2002;2:29–45.
46. von Bernhardi R, Ramirez G, Toro R, Eugenin J. Pro-inflammatory conditions promote neuronal damage mediated by amyloid precursor protein and decrease its phagocytosis and degradation by microglial cells in culture. Neurobiol Dis 2007;26:153–164.
47. Takahashi K, Rochford CD, Neumann H. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. J Exp Med 2005;201:647–657.
48. Saura CA. Presenilin/gamma-secretase and inflammation. Front Aging Neurosci 2010;2:16.