**SUMMARY**

Deciphering the mechanism by which the relative Aβ42/43 to total Aβ ratio is regulated is central to understanding Alzheimer disease (AD) etiology; however, the mechanisms underlying changes in the Aβ42(43) ratio caused by familial mutations and γ-secretase modulators (GSMs) are unclear. Here, we show in vitro and in living cells that presenilin (PS)/γ-secretase cleaves Aβ42 into Aβ38, and Aβ43 into Aβ40 or Aβ38. Approximately 40% of Aβ38 is derived from Aβ43. Aβ42(43) cleavage is involved in the regulation of the Aβ42(43) ratio in living cells. GSMs increase the cleavage of PS/γ-secretase-bound Aβ42 (increase kcat) and slow its dissociation from the enzyme (decrease koff), whereas PS1 mutants and inverse GSMs show the opposite effects. Therefore, we suggest a concept to describe the Aβ42(43) production process and propose how GSMs act, and we suggest that a loss of PS/γ-secretase function to cleave Aβ42(43) may initiate AD and might represent a therapeutic target.

**INTRODUCTION**

Alzheimer disease (AD) amyloid-β peptide (Aβ) and Aβ43 are generated from β-amyloid protein precursor (βAPP) and accumulate in senile plaques (Gravina et al., 1995). Because Aβ is secreted as multiple peptide species with different C termini, intramembrane proteolysis of Aβ by presenilin (PS)/γ-secretase (De Strooper et al., 1998, 1999; Sherrington et al., 1995; Struhl and Greenwald, 1999; Wolfe et al., 1999) does not occur at a unique site. However, because even small elevations in the ratio of Aβ42(43) to total Aβ (Aβ42/43 ratio) in secreted Aβ by PS or βAPP mutations trigger familial AD (Kuperstein et al., 2010; Scheuner et al., 1996; Suzuki et al., 1994), the proteolysis is regulated strictly in this aspect. How the variation in Aβ is generated remains unclear.

How βAPP-CTF is cleaved into Aβ42 is controversial because of conflicting findings. First, cleavage at the ε-site generates primarily long fragments, namely Aβ48 and Aβ49, and is followed by stepwise cleavage of every three amino acid residues starting at the C terminus (Qi-Takahara et al., 2005). Two distinct lines have been proposed for Aβ40 and Aβ42 production. Second, cleavage at the γ-site does not correlate directly with cleavage at the ε-site (He et al., 2010).

The ratio of APL1β28, a surrogate marker of Aβ42, to total APL1β is elevated in the cerebrospinal fluid of patients with sporadic AD, including those in the mild cognitive impairment stage and in those with familial AD (Yanagida et al., 2009). Thus, an increase in the Aβ42 ratio in the brain may play a role in the etiology of most AD cases, and the mechanism underlying the regulation of the Aβ42 ratio is a central issue in understanding AD.

γ-Secretase modulators (GSMs) are disease-modifying drugs that specifically reduce Aβ42 generation (Kounnas et al., 2010; Weggen et al., 2001); some GSMs are being studied in clinical trials. Despite the development of GSMs (Wolfe, 2012), their mechanism of action (Chávez-Gutiérrez et al., 2012) remains unclear.

Given that the VVIA peptide located between the γ38 and γ42 cleavage sites was detected in an in vitro βAPP-CTF cleavage assay (Takami et al., 2009), we asked whether Aβ42(43), formerly considered a product of PS/γ-secretase, could also be a substrate for PS/γ-secretase. Here, we show that Aβ42(43) is an intermediate of PS/γ-secretase, a finding that provides important insight into the regulation of the Aβ42(43) ratio. Our results will help elucidate the mechanism underlying the actions of GSMs and PS mutants.

**RESULTS**

**AD-Associated Aβ42 Is a Substrate of PS/γ-Secretase**

Initially, we performed a modified in vitro γ-secretase assay in which we used CHAPSO (3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonate) at its critical micelle concentration of 0.5% instead of 0.25%, the concentration used in the original method (Li et al., 2000). We experimented with using Aβ42 as a substrate instead of the βAPP-C-terminal fragment (βAPP-CTF) (Chávez-Gutiérrez et al., 2012). Surprisingly, Aβ38 was generated de novo from Aβ42 by PS/γ-secretase, a process that we refer to as “Aβ42 cleavage” (Figure 1). MALDI-TOF mass
Immunoprecipitation-immunoblot detection of Aβ. The reaction for immunoprecipitation was adjusted to contain the same amount of
levels of total Aβ cleavage assay.

Immunoprecipitation-MS analysis of products from the Aβ cleavage assay. See also Figures S1, S2, and S4.

Relative rate of the VVIA produced in (D), as defined by [VVIA]/[TVI]. The actual individual data from each of the three experiments are plotted. See also Figures S1, S2, and S4.

Figure 1. Detection of Aβ and Related Small Peptide Species
Aβ and jAPP-CTF cleavage assays were performed at 37°C for 30 min. (A) Immunoprecipitation-MS analysis of products from the Aβ and jAPP-CTF cleavage assay.

Next, we asked whether the Aβ cleavage process plays a role in conditions where the Aβ ratio in secreted Aβ changes in vitro and in living cells. First, we examined whether GSMs and inverse GSMs (iGSMs) (Kukar et al., 2005) change the rate of Aβ cleavage, when jAPP-CTF is the substrate. Cleavage of Aβ in the new assay was optimal at ~0.5% CHAPSO, whereas cleavage of jAPP-CTF in the conventional assay was optimal at ~0.2% CHAPSO (Li et al., 2000) (Figure 1C). Given this result, we considered the possibility that the Aβ ratio in the de novo Aβ generation in the jAPP-CTF cleavage assay might decrease at a higher CHAPSO concentration (~0.5%). As the CHAPSO concentration increased, the relative ratio of Aβ/APP-CTF production decreased and the ratio of Aβ to APP-CTF production increased (Figure 1D; Figure S2A). Surprisingly, the relative ratio of Aβ/APP-CTF to APP-CTF was ~0.1 at ~0.5% CHAPSO. Thus, we suspected that the much higher Aβ/APP-CTF ratio produced in the conventional jAPP-CTF cleavage assay than that secreted from living cells may be because the assay was performed in the presence of ~0.25% CHAPSO. In this regard, the in vitro γ-secretase assay in the presence of ~0.5% CHAPSO may be a better model.

Because Aβ levels are determined by the balance between its rate of production and its rate of degradation, we measured further the levels of small peptides produced by jAPP-CTF cleavage (Figure 1E). The relative amount of VVIA produced by jAPP-CTF cleavage increased as the Aβ ratio increased. These data suggest that the relative levels of Aβ are also a function of Aβ cleavage. Moreover, CTF-γ38/40/42/43, the counterparts of direct cleavage at the Aβ/APP-CTF sites, have never been observed (Gu et al., 2001). Collectively, our results indicate that Aβ is an intermediate stopover product of the cleavage of jAPP-CTF by PS/γ-secretase.

GSMs and Mutant PS1/γ-Secretases Increase and Decrease Aβ Cleavage in Living Cells, Respectively

Aβ42 and jAPP-CTF cleavage assays were performed at 37°C for 30 min.

Aβ42 and jAPP-CTF cleavage assays were performed at 37°C for 30 min. (B) Small Aβ-derived peptides (Takami et al., 2009) detected in the Aβ cleavage assay.

Next, we asked whether the Aβ cleavage process plays a role in conditions where the Aβ ratio in secreted Aβ changes in vitro and in living cells. First, we examined whether GSMs and inverse GSMs (iGSMs) (Kukar et al., 2005) change the rate of Aβ cleavage in vitro when the enzyme activity is low (0.25% CHAPSO) or high (0.5% CHAPSO). Strikingly, in the presence of 0.25% CHAPSO, all GSMs tested (GSM1, Eisai, Compound W, and Sulindac sulfide) increased the relative rate of Aβ cleavage (Figure 2A; Table S1A). In contrast, in the presence of 0.5% CHAPSO, the iGSMs tested (fenofibrate and
Figure 2. Effects of GSMs/iGSMs and Mutant PS1/γ-Secretases on Aβ42 Cleavage

(A) Fold activation of Aβ42 cleavage by GSMs/iGSMs in the presence of 0.25% CHAPSO. We extracted the PS/γ-secretase fraction from HEK cells stably expressing WT PS1 (Figure S2B). A total of 40 μM GSM1, 10 μM Eisai, 10 μM compound-W (CW), 10 μM SS, 50 μM fenofibrate (FF), or 10 μM S2474 was added to the in vitro assay.

(B) Fold activation of Aβ42 cleavage by GSMs/iGSMs in the presence of 0.5% CHAPSO.

(C) Fold changes of the relative VVIA levels in cell lysates treated with GSMs/iGSM. A total of 4 μM GSM1, 1 μM Eisai, or 30 μM S2474 was added to the cultured medium.

(D) The relative ratio of Aβ species in conditioned medium. The levels of Aβ38, Aβ40, and Aβ42 were measured by ELISA. Asterisks indicate p < 0.05, Welch's t test. Error bars represent SD. The actual individual data from each of the three experiments are plotted in (A), (B), and (F).

(E) Immunoblotting of purified PS/γ-secretase fractions. To show that the same level of each mutant PS1/γ-secretase was used in each reaction, we immunoblotted each fraction with antibodies against all four indispensable elements of PS1/γ-secretase: PS1/2, nicastrin, APh-1-a, and PEN-2. We detected almost equal band densities for all four proteins of mutants and WT PS1/γ-secretase fractions. Exogenous PS1 derivatives displaced the endogenous WT PS2 in the PS/γ-secretase complex. Note that a certain mutant contained a higher level of nicastrin, and thus was omitted from the analysis.

(F) Fold activation of Aβ42 cleavage by purified mutant PS1/γ-secretase.

(G) Fold changes of the relative VVIA levels in cell lysates stably expressing PS1 mutants.

(H) The relative ratio of Aβ species in conditioned medium.

The Aβ38 level was measured by ELISA. Asterisks indicate p < 0.05, Welch’s t test. Error bars represent SD. The actual individual data from each of the three experiments are plotted in (A), (B), and (F). See also Figures S1 and S2.
S2474) decreased the relative rate of Aβ42 cleavage (Figure 2B; Table S1B).

We do not know how CHAPSO affects the rate of Aβ42 cleavage in vitro. However, in the presence of 0.25% CHAPSO, when Aβ42 cleavage activity is low, iGSMs did not slow the reaction (Figure 2A). Similarly, GMSs did not increase Aβ42 cleavage in the presence of 0.5% CHAPSO, when the activity is high (Figure 2B). These findings suggest that the effects of CHAPSO and GMSs/iGSMs are related.

Next, we investigated whether an increase in Aβ42 cleavage by GMSs is responsible for the decrease in the Aβ42 ratio in living cells. We added GMSs and iGSMs to cultured HEK cells expressing wild-type (WT) and Swedish mutant (sw) βAPP stably, which were the same cell lines used for extracting PS1/γ-secretase for the in vitro experiments. We immediately boiled the treated cells for 2 min to inhibit completely the active degradation of tri-, tetra-, and pentaptides in the living cells. We extracted the soluble fraction from the resultant cell lysate and measured the levels of the small peptides by LC-MS/MS (Figures 2C and 2G).

Hereafter, the mention of an increase or a decrease in the levels of each peptide associated with the secretion of Aβ (e.g., VVIA, VVIAT, and IAT) implies that the relative ratio of a peptide was calculated in relation to that of the sum of peptides associated with Aβ generation (i.e., ITL, VIT, IVIV, TVI, IAT, VTVI, and VVIAT) (Figures 2, 3, and 4). GMS-treated cells, which secreted a lower ratio of Aβ42 (Figure 2D; Table S1D), contained a higher ratio of VVIA (produced by cleavage of Aβ42 into Aβ38) than did DMSO-treated cells (Figure 2C; Table S1C). In contrast, iGSM-treated cells, which secreted a higher ratio of Aβ42 (Figure 2D), contained a lower ratio of VVIA (Figure 2C). These data indicate that GMSs and iGSMs increase and decrease, respectively, the rate of Aβ42 cleavage in living cells.

Next, because the Aβ42 ratio in secreted Aβ is increased in most PS1 pathogenic mutants (Scheuner et al., 1996), we examined whether PS1 mutants integrated into the PS1/γ-secretase complex exhibit a reduced rate of Aβ42 cleavage. We extracted the PS1/γ-secretase fraction (Figure 2E; see also Figures S2F and S2B–S2D) (Winkler et al., 2009) from HEK cells stably expressing WT or mutant (L286V, L381V, or G384A) PS1 (Figures S2E and S2F). The same amount of enzyme complex was included in each reaction (Figure 2E). We found that the rate of Aβ42 cleavage was lower for mutant than for WT PS1/γ-secretase (Figure 2F; Table S1E), which is consistent with a previous report (Chávez-Gutiérrez et al., 2012). The results are reminiscent of the fact that cells expressing mutant PS1/γ-secretases generally secrete less total Aβ than do WT cells (Shen and Kelleher, 2007). However, it is of note that the reduced rate of Aβ42 cleavage described here is different from the overall loss of function of PS1/γ-secretase.

We investigated whether reducing Aβ42 cleavage by mutant PS1/γ-secretases could increase the Aβ42 ratio in living cells. We cultured WT or mutant PS1-expressing cells coexpressing sw βAPP (the same cell line used for the in vitro experiments) and analyzed the cell lysates by LC-MS/MS. The relative levels of VVIA were lower in the lysates of mutant cells than in WT cells (Figure 2G; Table S1F). We confirmed the increased Aβ42 ratio in the conditioned medium of the mutant-expressing cells (Figure 2H; Table S1F). These data demonstrate that mutant PS1/γ-secretases decrease the rate of Aβ42 cleavage in living cells. Collectively, our data suggest that the Aβ42 cleavage process is associated with the Aβ42 ratio in secreted Aβ.

**A New Concept for the Production of Bona Fide Aβ42**

To gain insight into the regulation of Aβ42 cleavage, we next examined how GMSs, iGSMs, and mutant PS1/γ-secretases alter Aβ42 cleavage activity in vitro. The conversion of Aβ42 into Aβ38 can be described by the following scheme and with the following rate constants:

\[
\text{PS1/γ-secretase} \rightarrow \left[ \text{Aβ42} \right] + \left[ \text{PS1/γ-secretase} / \right]
\]

This equation can be applied to the production of “free Aβ42” from de novo Aβ42 (shown in the diagram in Figure 3A). Escape from further cleavage and production of free Aβ42 both require that the de novo-generated bound Aβ42 dissociates from PS1/γ-secretase. We suggest this concept to explain the production of bona fide Aβ42. According to the model, \(k_{cat}\) (unimolecular rate constants) and \(k_b\) (dissociation rate constants) values would be relevant to the generation of free Aβ42.

One may think that once βAPP-CTF and PS1/γ-secretase form a complex, intermediate long Aβ does not dissociate from the enzyme during the stepwise cleavages. According to the model, the \(k_{cat}\) values of the Aβ42 cleavage should vary depending on the substrate. However, without any changes in the relative position of long Aβ species to PS1/γ-secretase, the stepwise cleavages might be interrupted. Moreover, we showed clearly that Aβ42, Aβ43, Aβ45, and Aβ46 can bind to PS1/γ-secretase and undergo cleavage (Figures 1 and 4). It is unknown whether all of the intracellular Aβ45 and Aβ46 (Qi-Takahara et al., 2005) bind to PS1/γ-secretase. Based on these findings, we suggest that the long Aβ species undergoes association/dissociation events with PS1/γ-secretase. Therefore, we revised the formulas describing the stepwise cleavage process proposed originally by Takami et al. (2009) (Figure 3B). We introduced the association/dissociation steps clearly for each cleavage step and did not consider that the cleavage at every three amino acid residues is an essential part of the cleavage process. Aβ42 and Aβ43 correspond to Aβ42n in the revised scheme. Aβ45 and Aβ46 correspond to Aβn in the revised scheme. Whether the various free Aβ products produced in each step remain at the membrane depends on their physicochemical nature. According to our model, the \(k_{cat}\) values of the Aβ42 cleavage should be the same, regardless of the substrate (e.g., βAPP-CTF and Aβ42).
Figure 3. Enzyme Kinetic Analysis of Aβ42 Cleavage and Biacore Analysis of Aβ42 Dissociation from PS1/γ-Secretase

(A) Proposed reaction mechanism for Aβ42 production.
(B) Proposed formulas of stepwise cleavage for Aβ production.
(C) Hanes-Woolf plots of the Aβ42 cleavage in the presence of 0.25% CHAPSO and GSMs (DMSO, black diamonds; GSM1, green squares; Eisai, blue triangles).

(legend continued on next page)
Using the RU values, we calculated $k_b$ values for the disso-
retase, which holds A142 cleavage (see Figures 2C and 2G; summarized in Figures 3I and 4I). The results indicate
that GSM1 and S2474 decreased and increased the rate of
dissociation of A142 from the active center of PS/γ-secretase
by 0.36 and 2.7 times, respectively, compared with the DMSO
control in the assay condition. We also performed similar experi-
ments to measure the relative $k_b$ values for the complex of A142
with WT and mutant PS1/γ-secretase (Figure 3J; Table S2H).
Next, we asked whether the relative level of A38 derived from A142 and that derived from A43 cleavage into A43 (i.e., VVIA and VVIAT) in living
cells was affected differently by the compounds and some
mutants. However, we are not yet able to show the extent of
the relative effects of the two factors (i.e., $k_{cat}$ and $k_b$) when
the A142 ratio changes in living cells.

**A GSM Reduces the Velocity at which Bound Aβ42 Dissociates from WT PS1/γ-Secretase, but the Complex of Mutant PS1/γ-Secretases and Aβ42 Dissociates Faster**

We also used Biacore binding analysis to measure the relative
dissociation rate constant $k_b$ for the complex of Aβ42 bound to
PS1/γ-secretase. Aβ42 was immobilized to the sensor tip, and purified PS1/γ-secretases were injected as the analytes. We
tried to measure the $k_b$ values to show how fast Aβ42 dissociates from the active center of PS/γ-secretase. We performed the Biacore assay with PS/γ-secretase preincubated in the presence or absence of L685,458, a transition state mimic that blocks the active site of PS/γ-secretase (see Figures S3E–S3J). We assumed that subtracting the resonance unit (RU) value for the L685,458–PS/γ-secretase complex binding to Aβ42 from the RU value for PS/γ-secretase alone (without mixing with L685,458) binding to Aβ42 would give the RU value for PS/γ-secretase, which holds Aβ42 in its active center (see Figures 3H and 3J). Using the RU values, we calculated $k_b$ values for the disso-
ciation of the bound Aβ42 from the active center.

We studied whether GSM1 or S2474 affects the $k_b$ value. During the period from 5 s to 155 s in the dissociation phase, the washout curves of compounds tested with Aβ42 showed simple one-step dissociation with the single exponential rate ex-
pected from the model (Figure 3H; Table S2F). The relative $k_b$
values of dissociation in the presence of GSM1 and S2474
were smaller and larger, respectively, than the value for WT
PS/γ-secretase alone (Figure 3I; Table S2G). These results suggest that the velocity at which bound Aβ42 dissociates
from PS1/γ-secretase contributes to the changes in the
Aβ42 ratio in secreted Aβ caused by the compounds and some
mutants. However, we are not yet able to show the extent of
the relative effects of the two factors (i.e., $k_{cat}$ and $k_b$) when
the Aβ42 ratio changes in living cells.

**Aβ43, Another Long Aβ Species, Is Cleaved into Aβ40 or Aβ38 by PS/γ-Secretase in Living Cells**

Aβ43 is another long species of Aβ (Saito et al., 2011). We also
investigated whether Aβ43 undergoes further proteolysis in a manner similar to Aβ42. MALDI-TOF MS (Figure 4A) and LC-
MS/MS (Figure 4B) showed that Aβ43 was cleaved to Aβ40 or Aβ38 by PS/γ-secretase in vitro. Thus, Aβ38 has multiple precur-
sors. This was confirmed by the in vitro APP-CTF cleavage assay (Figure 4C). Why PS/γ-secretase cleaved the substrate
at only one of two sites remains unclear. The production of Aβ37 and GVV indicates minor but further cleavage of de novo
Aβ40 (Figures 4A and 4B; Figures S1C and S1D).

We measured the amounts of tri-, tetra-, and pentapeptides produced during the stepwise processing of APP in living cells
(Figure 4D; Figure S4A). Approximately 40% of Aβ38 was derived from Aβ43 (Table S3A). GSMs increased the relative rate of Aβ43 cleavage into Aβ38 (i.e., the level of VVIA relative to that of total Aβ-related small peptides) (Figure 4E; Table S3B), whereas iGSM and mutant PS1/γ-secretases decreased the rate (Figure 4F; Table S3C). These results are very similar to the effects of the compounds and the mutants on Aβ42 cleavage (see Figures 2C and 2G; summarized in Figures 4I and 4J). However, both the tested GSM/iGSM (Figure 4G; Table S3D) and some mutant PS1/γ-secretases decreased the rate of Aβ43 cleavage into Aβ40 (i.e., IAT). Thus, the cleavage of Aβ43 into Aβ38 and that into Aβ40 were affected differently by
GSMs.

Next, we asked whether the relative level of Aβ38 derived from Aβ42 and that derived from Aβ43 (i.e., VVIA and VVIAT) in living

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(D) Hanes-Woolf plots of Aβ42 cleavage in the presence of 0.5% CHAPSO and iGSM (DMSO, black diamonds; S2474, red squares).
(E) The relative $k_{cat}$ values (n = 4) of Aβ42 cleavage in the presence of GSMs/GSM.
(F) Hanes-Woolf plots of Aβ42 cleavage by WT and mutant PS1/γ-secretase (WT PS1, black diamonds; PS1 L286V, red triangles; PS1 L381V, blue squares; PS1 G384A, purple crosses) in the presence of 0.5% CHAPSO.
(G) The relative $k_b$ values of Aβ42 cleavage by WT and mutant PS1/γ-secretase.
(H) Fitted curves of dissociation for DMSO (purple/black), GSM1 (18 μM, green/grey), and S2474 (135 μM, blue/red).
(I) $k_b$ values (n = 3 for each) in the presence of GSM/iGSM treatment compared with those obtained in the presence of DMSO treatment.
(J) Fitted curves of dissociation (WT PS1/γ-secretase, blue/black; PS1 L286V/γ-secretase, purple/red; PS1 G384A/γ-secretase, green/grey).
(K) $k_b$ values of mutant PS1/γ-secretases relative to those of WT PS1/γ-secretase. Asterisks indicate $p < 0.05$, Welch’s t test. Error bars represent SD. See also Figure S3.
Long Aβ-like peptides other than Aβ are also substrates of PS/γ-secretase

Aβ-like peptides (Okochi et al., 2002), secreted by a process similar to that for Aβ secretion, include mNotch-1-derived Nβ (Okochi et al., 2006) and APLP1-derived APL1 (Yanagida et al., 2009). We found that PS/γ-secretase cleaved Nβ25 into Nβ21 (Figure 4M) and APL1β28 into APL1β25 (Figure 4N), suggesting that long Aβ-like peptides are generally intermediate products. This may explain why the relative levels of some longer secreted Aβ-like peptides, including Aβ42, change in parallel (Okochi et al., 2006; Yanagida et al., 2009). This finding also indicates that APL1β28 cleavage to APL1β25 is impaired in the sporadic AD brain (Yanagida et al., 2009).

DISCUSSION

In this study, we show that de novo Aβ42(43), a secreted species, is an intermediate of PS/γ-secretase in living cells, and this discovery affects the understanding of the nature of Aβ42(43) production. We suggest that Aβ42 production does not directly reflect the level of cleavage at the C terminus of Aβ42, but rather depends on how much newly produced Aβ42 dissociates from the PS/γ-secretase enzyme and thereby avoids further cleavage. Thus, competition between further cleavage and dissociation from the enzyme may be the key to determining the Aβ42(43) ratio. Importantly, our results also suggest that a new type of partial loss of function in PS/γ-secretase [e.g., reduction in Aβ42(43) cleavage or at the final step of PS/γ-secretase cleavage of AβAPP] may cause a gain of function in AD [an increase in the Aβ42(43) ratio]. GSMS increase the relative kcat for the further cleavage of Aβ42 to Aβ38 and decrease the relative kcat for the dissociation of Aβ42 from PS1/γ-secretase. This suggests a potential model to explain how GSMS can lower Aβ42 production.

Chávez-Gutiérrez et al., 2012 showed that PS1 mutations lower the relative levels of Aβ38 to Aβ42 and Aβ40 to Aβ43 compared with WT PS1. The GSMS tested increased both the level of Aβ38 relative to Aβ42 (Weggen et al., 2001) and the level of Aβ40 relative to Aβ43. Based on the hypothetical model proposed by Ihara and colleagues (Takami et al., 2009), those authors speculated that the PS1 mutants and GSMS decrease and increase, respectively, the rate of the fourth cleavage (i.e., Aβ43 cleavage to Aβ40 and Aβ42 cleavage to Aβ38, respectively), possibly because of the premature release of the Aβ42/Aβ43 peptides.
We found that, in living cells, ~40% of Aβ38 was derived from Aβ43. Moreover, Aβ40 and Aβ41 were produced in the Aβ45 cleavage assay, and Aβ42 was produced in the Aβ46 cleavage assay. Thus, the putative Aβ38 and Aβ40 product lines (Takami et al., 2009) turn out to overlap at several points.

Takami et al. (2009) showed that Sulindac sulfide decreased the levels of Aβ42 and Aβ43 in a βAPP-CTF cleavage assay, but did not significantly increase the levels of VVIA and IAT. This may be because Sulindac sulfide exerts a weaker GSM action than GSM1 and Elsail, which were used in this study.

We also found that Notch-1 and APLP1 transmembrane domains are cleaved in a similar way, which should help clarify the physiological process of intramembrane proteolysis by PS/γ-secretase. We showed previously that Aβ42 ratio changed in parallel with APL1β28 ratio, and that the APL1β28 ratio increases in the cerebrospinal fluid of AD patients (Yanagida et al., 2009). In this article, we demonstrated that both Aβ42 and APL1β28 were cleaved similarly into the shorter species (i.e., Aβ38 and APL1β25). Therefore, we suggest that Aβ42 cleavage may also decrease in AD brains.

Collectively, we speculate that the increase in the Aβ42 ratio simply reflects the accelerated dissociation of membrane-bound long Aβs (i.e., Aβ44–49) from PS/γ-secretase. Because long Aβs on the membrane may perturb the physiological function of neuronal cells, further studies are necessary to investigate whether the prolonged stay of long Aβ at the membrane is pathologically relevant.

At present, inhibition of PS/γ-secretase activity using agents such as Notch-inhibiting spars is the central approach to decreasing Aβ production specifically. Our results may shift the nature of new drugs for treating AD to repair or increase the ability of PS/γ-secretase to cleave Aβ42(43).

**EXPERIMENTAL PROCEDURES**

Aβ and Aβ-like Peptide Cleavage Assays
In vitro γ-secretase assays (Li et al., 2000; Osawa et al., 2008) using Aβ and Aβ-like peptides (Aβ42, 43, 45, 46, Nι25, and APL1β28) were performed under the modified conditions described here with a modified reaction buffer (150 mM citrate buffer [pH 6.0], 0.25 M sucrose, 0.04%–1.5% CHAPSO, the modified conditions described here with a modified reaction buffer A

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cebrep.2012.11.028.

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