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# Phenylpiperidine-type γ-secretase modulators target the transmembrane domain 1 of presenilin 1

**Yu Ohki[1], Takuya Higo[2], Kengo Uemura[3], Naoaki Shimada[2], Satoko Osawa[1], Oksana Berezovska[3], Satoshi Yokoshima[2], Tohru Fukuyama[2], Taisuke Tomita[1,4,*] and Takeshi Iwatsubo[1,4,5,*]**

[1]Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan, [2]Department of Synthetic Natural Products Chemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan, [3]Alzheimer Research Unit, MassGeneral Institute for Neurodegenerative Disease, Massachusetts General Hospital, Charlestown, MA, USA, [4]Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Tokyo, Japan and [5]Department of Neuropathology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

**Amyloid-β peptide ending at the 42nd residue (Aβ42) is implicated in the pathogenesis of Alzheimer's disease (AD). Small compounds that exhibit selective lowering effects on Aβ42 production are termed γ-secretase modulators (GSMs) and are deemed as promising therapeutic agents against AD, although the molecular target as well as the mechanism of action remains controversial. Here, we show that a phenylpiperidine-type compound GSM-1 directly targets the transmembrane domain (TMD) 1 of presenilin 1 (PS1) by photoaffinity labelling experiments combined with limited digestion. Binding of GSM-1 affected the structure of the initial substrate binding and the catalytic sites of the γ-secretase, thereby decreasing production of Aβ42, possibly by enhancing its conversion to Aβ38. These data indicate an allosteric action of GSM-1 by directly binding to the TMD1 of PS1, pinpointing the target structure of the phenylpiperidine-type GSMs.**

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## Introduction

There is ample evidence supporting the notion that aggregation of amyloid-β peptides (Aβ) underlies the pathogenesis of Alzheimer's disease (AD) (reviewed by Holtzman *et al*, 2011). Aβ is a proteolytic fragment of amyloid precursor protein (APP) derived by sequential cleavages by two proteases termed β- and γ-secretases. γ-Secretase determines the C-terminal length of Aβ, which in turn impacts the aggregation property of Aβ: the C-terminally longer Aβ42 is the initially deposited and the most aggregation-prone species linked to the pathogenesis of AD (Iwatsubo *et al*, 1994). Thus, small compounds that regulate the activity of γ-secretase have been developed as disease-modifying drugs against AD (Tomita, 2009; De Strooper *et al*, 2010). However, conventional γ-secretase inhibitors (GSIs) that block the proteolytic cleavage of numerous substrate proteins in a non-specific fashion cause severe adverse effects. For example, GSIs interfere with Notch receptor signalling that is essential to development and differentiation, and is activated by the γ-secretase cleavage (Extance, 2010). The discovery that a subset of non-steroidal anti-inflammatory drugs (NSAIDs) act as a γ-secretase modulator (GSM) that selectively and directly reduces Aβ42 without affecting the total γ-secretase activity opened up a way to the safe and effective lowering of the pathogenic Aβ42 while sparing the Notch signalling (Weggen *et al*, 2001; Takahashi *et al*, 2003). Although the clinical trial of Flurizan (*R*-Flurbiprofen), an NSAID-type GSM, failed because of the low penetrance of the compound into the brain (Green *et al*, 2009), several potent GSMs with excellent brain availability are emerging, raising high hopes for the treatment of AD (Tomita, 2009; De Strooper *et al*, 2010).

γ-Secretase is an atypical intramembrane protease that comprising four integral membrane proteins, that is, presenilin (PS), nicastrin, Aph-1 and Pen-2 (Takasugi *et al*, 2003). PS serves as the catalytic subunit of γ-secretase, in which a pair of aspartate residues located on the N- and C-terminal endoproteolytic fragments of PS (NTF and CTF, respectively) comprise the catalytic site. A series of chemical biology experiments revealed that all GSIs investigated so far directly target PS (Tomita, 2009). However, the molecular target, as well as the mechanism of action, of GSMs remains enigmatic. A couple of recent studies suggested that NSAID-based GSMs directly and specifically target APP, but not the enzyme (i.e., γ-secretase; Kukar *et al*, 2008). In contrast, several lines of evidence suggest that the effects of GSM are not limited to the γ-secretase cleavage of APP: it has been shown that some GSMs modulated the C-terminal length of Nβ, a proteolytic product of Notch corresponding to Aβ (Okochi *et al*, 2006), and the activity of signal peptide peptidase (SPP), a PS-type intramembrane cleaving protease with an inverse orientation of the catalytic structure, also was affected by NSAIDs (Sato T *et al*, 2006). Moreover, reports casting doubt to the substrate targeting of NSAIDs have recently been published (Beel *et al*, 2009; Page *et al*, 2010). However, there has not been direct and conclusive evidence regarding the target of the GSMs.

Here, we examined the target and mode of action of GSM-1, one of the representative Aβ42-lowering GSMs harbouring a phenylpiperidine structure (Page *et al*, 2008). Using two novel photoprobes derivatized from GSM-1, we have unequivocally located the binding site of the phenylpiperidine-type GSM on a subdomain within the transmembrane domain (TMD) 1 of PS1, which has been shown to participate in the catalytic pore structure of γ-secretase (Takagi *et al*, 2010).

*Corresponding authors. T Tomita or T Iwatsubo, Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Tel.: +81 3 5841 4895; Fax: +81 3 5841 4708; E-mail: taisuke@mol.f.u-tokyo.ac.jp or iwatsubo@m.u-tokyo.ac.jp

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Our results suggest that GSM-1 decreases the production of Aβ42 possibly by enhancing its conversion to Aβ38, by an allosteric mechanism by binding to the TMD1 of PS1.

## Results

### *GSM-1 affects the Aβ38- and Aβ42-generating activities of γ-secretase*

GSM-1 is a phenylpiperidine-type compound that shows a potent Aβ42-lowering effect ($IC_{50}=0.348\,\mu M$ in a cell-based assay) accompanied by an increased production of Aβ38 (Figure 1A), as documented (Page *et al*, 2008). Recent biochemical studies suggest that γ-secretase executes a stepwise removal of tripeptides from the C termini of longer Aβ species that are cognate precursors for secreted form of Aβ: Aβ40 and Aβ42 are successively derived from Aβ43/46/49 and Aβ45/48, respectively (Qi-Takahara *et al*, 2005; Takami *et al*, 2009). An additional cleavage of Aβ42 generates Aβ38. To further characterize the effect of GSM-1 on the stepwise γ-secretase cleavage leading to Aβ secretion, we examined the production of the different Aβ species (i.e., Aβ38, Aβ40, Aβ42, Aβ43, Aβ45 and Aβ46) in an *in-vitro* assay. The levels of total Aβ and its C-terminal counterpart product, APP intracellular domain (AICD), were not affected by GSM-1 treatment (Supplementary Figure S1A–C). However, GSM-1 selectively reduced the Aβ42 generation accompanied by an increase in Aβ38, similarly to the results in the cell-based assay (Figure 1B and C). Notably, generation of Aβ45, a hypothetical precursor of Aβ42 and Aβ38, was not affected. These data suggest that GSM-1 exclusively affected the γ-cleavage that cuts the midst of the TMD of substrates, but not the ε- or ζ-cleavages (corresponding to the production of AICD/Aβ48/49 and Aβ45/46, respectively) that occur at positions closer to the cytoplasm. To ascertain that GSM-1 directly affects the γ-cleavage, we examined the effect of GSM-1 in an *in-vitro* γ-secretase assay using reconstituted γ-secretase complex recovered from Sf9 cells infected with recombinant baculovirus (Hayashi *et al*, 2004; Ogura *et al*, 2006). Again, GSM-1 caused a decrease in the *de novo* generation of Aβ42 and an increase in that of Aβ38, confirming the direct action of GSM-1 on the γ-secretase-mediated cleavage (Figure 1D).

### *GSM-1 directly binds to the N-terminal fragment of PS1*

To identify the molecular target of GSM-1, we employed photoaffinity labelling (PAL; Morohashi *et al*, 2006; Fuwa *et al*, 2007) using photoactivatable probes harbouring photoactivatable and biotin moieties. The molecular target of the probe is covalently crosslinked upon UV irradiation and purified by the avidin-biotin catch principle (Hofmann and Kiso, 1976). We synthesized a GSM-1-based photoactivatable probe, GSM-1-BpB (see Supplementary Scheme 1), in which

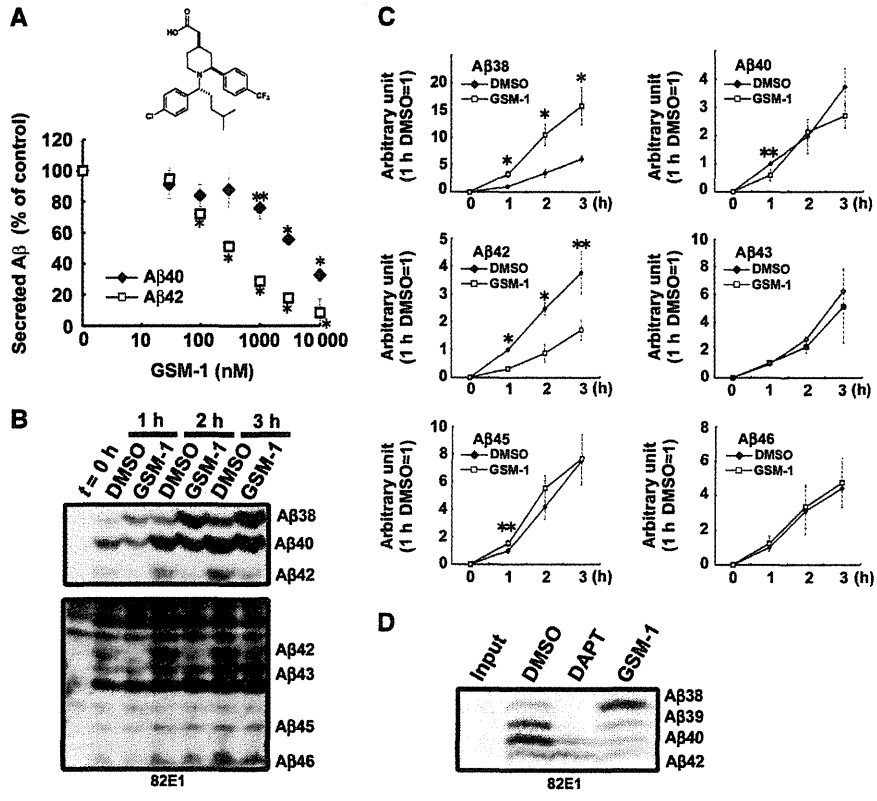


**Figure 1** GSM-1 directly and selectively affects the Aβ38/42-generating γ-secretase activity. (**A**) Chemical structure of GSM-1 and its pharmacological effect on a cell-based assay. Aβ levels in conditioned media from HEK293 cells were analysed by ELISAs ($n=3$, mean ± s.d., $^*P<0.01$, $^{**}P<0.05$ at Student's *t*-test). (**B**) Effect of GSM-1 (25 μM) on stepwise cleavage by γ-secretase in an *in-vitro* assay. *De novo* generated Aβ species were separated by two types of gels (Qi-Takahara *et al*, 2005). (**C**) Quantitation of *de novo* generated Aβ in an *in-vitro* assay ($n=3$, mean ± s.d., $^*P<0.01$; $^{**}P<0.05$ at Student's *t*-test). (**D**) *De novo* Aβ generation using purified γ-secretase in the presence of DAPT (20 μM) or GSM-1 (20 μM).

 

benzophenone and biotin moieties were conjugated to GSM-1. GSM-1-BpB retained the Aβ42-lowering ($IC_{50}$ = 3.82 μM in a cell-based assay) and Aβ38-raising activities (Figure 2A and B). Neither GSM-1 nor GSM-1-BpB affected the production of NICD, suggesting that GSM-1-BpB harbours a similar modulator effect to GSM-1 (Figure 2C). Next, we performed the PAL experiments by GSM-1-BpB in mouse brain microsomes, and detected the specific biotinylation of endogenous PS1 NTF, which was decreased by co-incubation with the parent compound GSM-1 (Figure 3A and B). We did not observe labelling of other γ-secretase components, nor of the APP C-terminal stub, by GSM-1-BpB. Specific labelling of PS1 by GSM-1-BpB was also observed in immortalized fibroblasts derived from *Psen1*$^{-/-}$/*Psen2*$^{-/-}$ mice (DKO cells) (Herreman *et al*, 2000) stably expressing His-tagged PS1, supporting the direct targeting of GSM-1 to PS1 NTF (Figure 3C). Moreover, recombinant SPP (Sato T *et al*, 2006; Fuwa *et al*, 2007), a PS family protease, also was directly targeted by GSM-1 and underwent specific modulation of its activity (Supplementary Figure S2A and B). $(Z\text{-}LL)_2$-ketone, well-known SPP-specific inhibitor, unaffected SPP labelling by GSM-1-BpB. Collectively, these data supported the notion that GSM-1 is directly bound to PS-type intramembrane cleaving proteases, and that PS1 NTF is a *bona fide* target of GSM-1.

### *Phenylpiperidine-type GSMs affect the structure of PS1 NTF in an allosteric manner*

Next, we performed cross-competition experiments in PAL using a set of conventional GSIs to examine the effect of GSM-1 binding on the enzymatically functional sites within PS1. We used L-685,458, pep.11 and compound E, which are predicted to target γ-secretase catalytic site, the initial substrate binding site and the transit path between these sites, respectively, as competitors (Li *et al*, 2000; Das *et al*, 2003; Fuwa *et al*, 2007). To ensure a fair competition with the GSM-1-BpB binding, we employed pretreatment with unlabelled competitor compounds at 10-fold concentrations of the $IC_{50}$ values against Aβ42 generation ($IC_{50}$ values: GSM-1, 24.09 μM (Supplementary Figure S3A and B); L-685,458, 0.59 μM; pep.11, 0.48 μM; compound E, 0.97 nM as determined in our cell-free γ-secretase assays). Pretreatment with neither of these three GSIs affected the labelling of PS1 NTF by GSM-1-BpB (Figure 4A). Furthermore, 10 μM of each GSI also unaffected GSM-1-BpB binding (Supplementary Figure

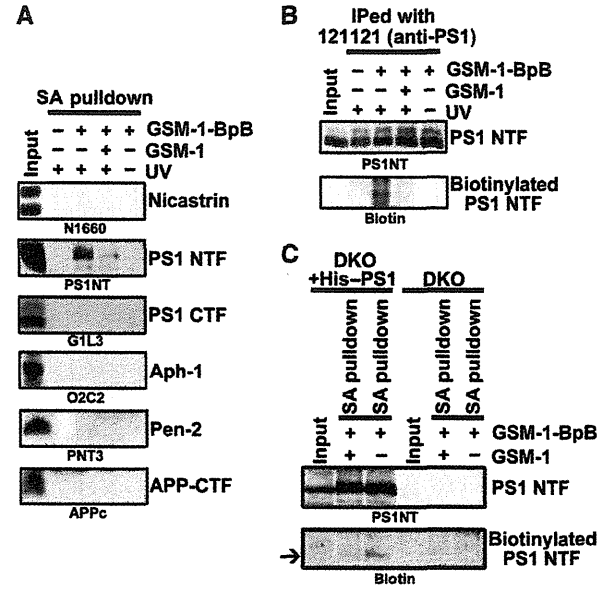


**Figure 3** GSM-1-BpB directly binds to PS1 NTF. (**A**) PAL experiments using mouse brain membranes. PS1 NTF was specifically labelled with GSM-1-BpB (1 μM). The labelling was competed by preincubation with GSM-1 (100 μM). (**B**) Direct biotinylation of PS1 NTF. After PAL, PS1 NTF was precipitated using anti-PS1 NTF antibody and analysed by an anti-biotin antibody. (**C**) Biotinylation of His-tagged PS1 expressed in DKO cells by GSM-1-BpB. After PAL, His-tagged PS1 (*arrow*) was purified with $Ni^{2+}$-affinity column and analysed by immunoblotting.

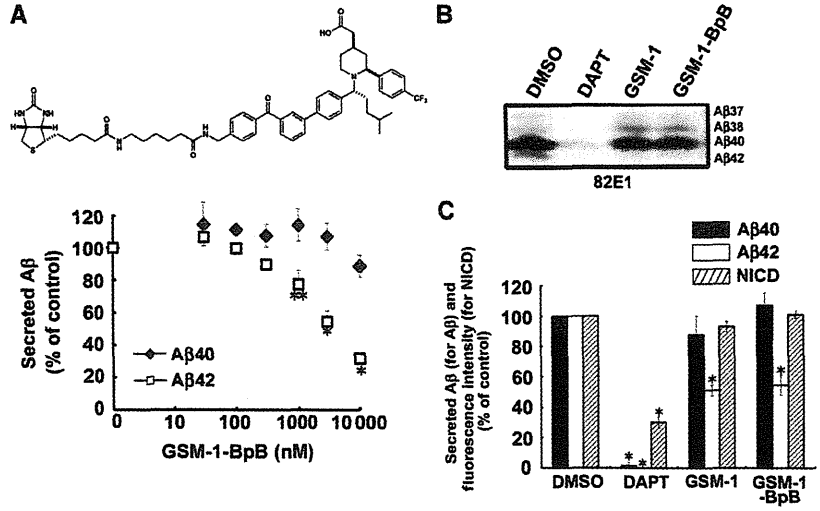


**Figure 2** GSM-1-BpB harbours Aβ42-lowering modulator activity. (**A**) Chemical structure of GSM-1-BpB and its pharmacological effect on a cell-based assay ($n$ = 3, mean ± s.d., *$P$<0.01, **$P$<0.05 at Student's $t$-test). (**B**) Immunoblotting analyses of conditioned media from HEK293 cells in the presence of DAPT (10 μM), GSM-1 (2 μM) or GSM-1-BpB (2 μM). (**C**) Effects on Notch cleavage of DAPT (10 μM), GSM-1 (0.3 μM) or GSM-1-BpB (3 μM) ($n$ = 3, mean ± s.d., *$P$<0.01, **$P$<0.05 at Student's $t$-test).

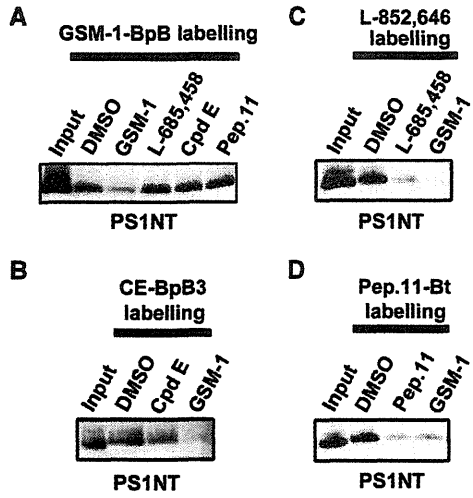


**Figure 4** Competition of the photoaffinity labelling of PS1 NTF by GSM-1-BpB. (**A**) Labelling competition experiment with conventional GSIs for the labelling of PS1 NTF by GSM-1-BpB. GSIs were tested at the 10-fold concentration of $IC_{50}$ (final concentrations: compound E (Cpd E) (9.7 nM), L-685,458 (5.9 μM) and pep.11 (4.8 μM)). (**B–D**) Labelling competition experiments with GSM-1 (240.9 μM) for the labelling of PS1 NTF by CE-BpB (**B**), L-852,646 (**C**) and pep.11-Bt (**D**).

S4A), indicating that the binding site of GSM-1 is distinct from those of the conventional GSIs. In contrast, pretreatment with GSM-1 significantly inhibited the labelling of PS1-NTF by CE-BpB3, L-852,646 and pep.11-Bt, which are photoprobes derived from compound E, L-685,458 and pep.11, respectively (Figure 4B–D). Importantly, GSM-1 showed no competition on the labelling by DAP-BpB, which has been known to target PS1 CTF (Morohashi *et al*, 2006), ensuring the specificity of the competition by GSM-1 (Supplementary Figure S4B). We further examined the effects of γ-secretase substrates on GSM-1-BpB labelling, since other classes of GSMs (i.e., ibuprofen and fenofibrate) have been suggested to dock with PS in the presence of substrates (Uemura *et al*, 2010). Notably, labelling of GSM-1-BpB was altered neither by pretreatment with a substrate-mimetic inhibitor pep.11 (Figure 4A), nor by overexpression of C99, an APP-based substrate (Supplementary Figure S5), indicating that GSM-1 specifically bound to PS1 NTF independent of substrate binding. These data support the notion that binding of GSM-1 to PS1 NTF affects the structures of enzymatically functional sites within PS1.

Through the structure–activity relationship analysis of GSM-1, we found that the carboxylic acid moiety is critical for the Aβ42-lowering effect. Among the derivatives, NS-1017 (See Supplementary Scheme 2), which harbours an *N*-benzylamide moiety showed a significant Aβ42-raising effect associated with decreased Aβ38 production (Figure 5A and B). Intriguingly, preincubation with NS-1017 diminished the labelling of PS1 NTF by GSM-1-BpB (Figure 5C), suggesting that the phenylpiperidine structure is the pharmacophore that targets the modulator binding site within PS1 NTF, and that the substituent at the 4-position of the piperidine (i.e., carboxylic acid or amide) determines the pharmacological effects on Aβ42 production. We also synthesized a phenylpiperidine-type Aβ42-raising photoprobe, GSM-1-amide-BpB (See Supplementary Scheme 3), in which the biotin-benzo-

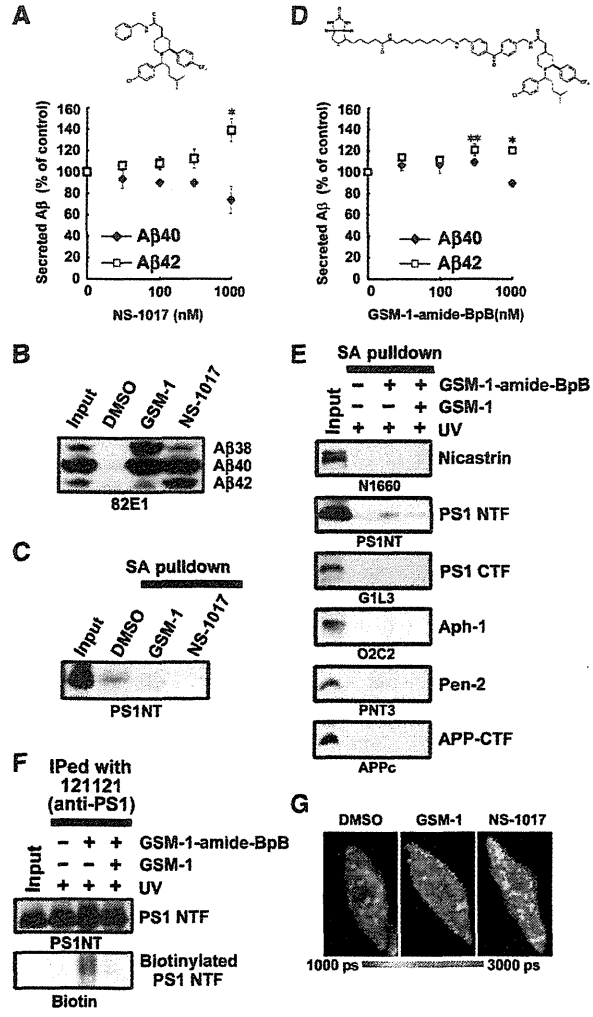


**Figure 5** Aβ42-raising phenylpiperidine-type modulator directly targets PS1 NTF. (**A**) Chemical structure of NS-1017 and its pharmacological effect on a cell-based assay ($n=3$, mean ± s.d., $^*P<0.01$ at Student's *t*-test). (**B**) Immunoblot analysis of secreted Aβ in conditioned media in the presence of DAPT (10 μM), GSM-1 (2 μM) or NS-1017 (2 μM). (**C**) Labelling competition experiment with GSM-1 (100 μM) or NS-1017 (200 μM) for the GSM-1-BpB labelling of PS1 NTF (1 μM) using mouse brain microsomes. (**D**) Chemical structure of GSM-1-amide-BpB and its pharmacological effect on a cell-based assay ($n=3$, mean ± s.d., $^*P<0.01$, $^{**}P<0.05$ at Student's *t*-test). (**E**) PAL experiment using GSM-1-amide-BpB (1 μM) in mouse brain microsomes. (**F**) Immunoprecipitated PS1 NTF was analysed by an anti-biotin antibody after PAL in mouse brain microsomes. (**G**) Effects of GSM-1 and NS-1017 on the structure of PS1 revealed by FLIM assay. Pseudo-coloured FLIM images showed subcellular distribution of the GFP lifetimes, with red pixels representing shorter lifetime (closer GFP-PS1 NT and RFP-PS1-loop proximity). A colorimetric scale bar shows colour-coded fluorescence lifetime in picoseconds.

phenone moiety was directly connected to the amide moiety (Figure 5D). GSM-1-amide-BpB also specifically biotinylated PS1 NTF in mouse brain membranes, supporting the notion that the phenylpiperidine structure is required for the targeting to the modulator binding site (Figure 5D and E).

Using fluorescence lifetime imaging microscopy (FLIM), we have previously reported that the mature PS1 within the active γ-secretase complex exists in equilibrium between two conformational states: 'open' and 'closed' NTF/CTF confor-

 

**Table I** Differences in the proximity between N- and C-termini of PS1 shown by FLIM assay

| Construct | Compound | Av lifetime | s.d. | *n* | *t*-test versus PS1 DMSO |
|---|---|---|---|---|---|
| GFP-PS1-RFP | DMSO | 1872.4 | 144.3 | 61 | — |
| GFP-PS1-RFP | GSM-1 | 1921.0 | 114.0 | 54 | 0.0463* |
| GFP-PS1-RFP | NS-1017 | 1812.4 | 154.1 | 71 | 0.0388* |

*$P<0.05$ versus GFP-PS1-RFP DMSO (Student's *t*-test).

mations that correspond to the low and high ratio of Aβ42/Aβ40 production, respectively (Lleo *et al*, 2004; Uemura *et al*, 2009). By FLIM, we found that GSM-1 treatment leads to a significant increase in the GFP lifetime in cells expressing PS1 fused with GFP and RFP at the N-terminus and within large hydrophilic loop of PS1 CTF, respectively. This indicates that GSM-1 treatment caused an 'open-form' PS1 conformation similar to that caused by Aβ42-loweirng NSAID-type GSMs. Interestingly, NS-1017 had an opposite effect on PS1, and similarly to fenofibrate led PS1 to exhibit the 'close-form' conformation (Figure 5G; Table I; Lleo *et al*, 2004; Uemura *et al*, 2009). These data indicate that the phenylpiperidine-type GSMs modulated γ-cleavage through conformational changes of PS1 by interacting with a common binding site.

### *The TMD1 of PS1 is required for the binding of GSM-1*

FLIM results suggested a conformational change of PS1 that results in altered proximity between the N-terminus and loop domain of PS1. However, we found that PS1 mutant lacking the N-terminal amino-acid residues from Thr2 to Ala79 (PS1Δ79) retained the sensitivity to GSM-1 in DKO cells (Supplementary Figure S6A–C). In addition, phenylpiperidine-type GSMs were effective on γ-secretase complex containing PS2 with a low sequence similarity to PS1 at the N-terminus (Supplementary Figure S6D and E). These data indicated that the N-terminal cytoplasmic domain of PS1 is dispensable to the pharmacological effect of GSM-1. To further narrow down the binding site of GSM-1 within the PS1 NTF, we have taken a limited digestion approach: we inserted a thrombin substrate sequence (i.e., LVPRGS) into the hydrophilic regions within PS1 NTF (i.e., PS1-Th60N at the N-terminus, PS1-Th1 and PS1-Th3 at the loops 1 and 3, respectively; Figure 6A), and confirmed that overexpression of these mutants in DKO cells restored the γ-secretase activity that was modulated by GSM-1 (Figure 6B; Supplementary Figure S7). Proteolytic cleavage of PS1-Th60N, PS1-Th1 and PS1-Th3 mutants by thrombin resulted in generation of 8, 12 and 23 kDa N-terminal fragments of PS1 NTF, respectively, which are detectable by the anti-PS1 N-terminus antibody (Figure 6C). To pinpoint the modulator binding domain within PS1 NTF, we subjected these mutants to thrombin treatment after PAL with GSM-1-BpB (Figure 6D). N-terminal fragments derived from PS1-Th1 or PS1-Th3 were pulled down by streptavidin beads, but that from PS1-Th60N was not (Figure 6D). Furthermore, preincubation with GSM-1 reduced the labelling of the cleaved fragment of PS1-Th1 (Figure 6E). These data suggest that the GSM-1 binding site resides within the region between Lys80 and Asp110.

Using CHO cells in which holoprotein form of endogenous PS1 was detectable, we found that GSM-1-BpB and GSM-1-amide-BpB specifically labelled the PS1 holoprotein (Supplementary Figure S8A and B). PS1 mutant carrying the protease-inactive D385A mutation also was labelled. These data suggest that GSM-1 binds to PS1 irrespective of the formation of the stable γ-secretase complex or the proteolytic activity. Taking advantage of these features, we examined the labelling of TMD-swap mutants of PS1, in which each TMD was replaced with that of an unrelated transmembrane protein, CLAC-P, with a proper orientation. These TMD-swap mutants failed to exhibit the enzyme activities, although forming the γ-secretase complex (Watanabe *et al*, 2005, 2010). TM1mt PS1, in which amino-acid residues Val82 to Ile100 of PS1 were replaced, failed to be labelled by GSM-1-BpB, whereas TM5mt and TM9mt PS1 were biotinylated (Figure 6F). To further verify the specificity of binding of GSM-1-BpB to the TMD1 of PS1, we subjected purified recombinant glutathione S-transferase (GST), GST fused protein of $PS1_{2-65}$ or $PS1_{1-110}$ to PAL with GSM-1-BpB (Figure 6G; Supplementary Figure S9). Notably, photolysis of GSM-1-BpB resulted in the specific binding exclusively to GST-$PS1_{1-110}$ encompassing the TMD1, but not to GST-$PS1_{2-65}$ corresponding to the N-terminal cytoplasmic portion of PS1, indicating the requirement of TMD1 for the binding of GSM-1-BpB (Figure 6H). Collectively, these data suggest that TMD1 is the binding site of phenylpiperidine-type GSMs.

### *GSM-1 targets the hydrophobic portion of the TMD1 of PS1 and allosterically affects the conformation of PS1*

To further characterize the structural changes of TMD1 of PS1 caused by binding of GSM-1, we utilized substituted cysteine accessibility method (SCAM), a biochemical analysis of the structures of membrane proteins in a membrane-embedded, functional state, by assessing the hydrophilicity of each amino-acid residue by the biotinylation efficiency of mutated cysteine at the same position. We have previously shown that TMD1 of PS1, comprising residues Gly78 to Ile100, faces a hydrophilic environment within the membrane by SCAM (Takagi *et al*, 2010). TMD1 consists of two functional regions: the N-terminal portion close to the cytoplasm is facing the catalytic site of the γ-secretase, whereas the C-terminal portion is buried within the lipid bilayer and possibly involved in the structural integrity of PS1 via hydrophobic interactions with other TMDs. The results of PAL and FLIM experiments suggested that a conformational change of TMD1 was linked to the modulator activity of GSMs. Intriguingly, mutant PS1 replaced with the amino-acid sequences of CLAC-P at this hydrophilic segment (i.e., Val82 to Pro88) retained the γ-secretase activity as well as the sensitivity to the modulatory effect of GSM-1, suggesting that the C-terminal side, rather than the N-terminal side, of TMD1 harbours the modulator binding site (Supplementary Figure S10A–C). These observations further prompted us to analyse the effect of GSM-1 on the water accessibility of the six residues within TMD1 (i.e., Lys80, His81, Val82, Ile83, Met84 and Leu85), which directly face the hydrophilic environment by SCAM. We have previously shown that competition of SCAM labelling is useful for the identification of the binding site, or the allosteric effect, of small compounds (Sato C *et al*, 2006, 2008; Takagi *et al*, 2010). Treatment with either GSM-1 or NS-1017 slightly, but constantly reduced the labelling of single-cysteine PS1 mutant at Met84 (M84C), whose labelling has never been affected by competition with conventional GSIs (Takagi *et al*, 2010). In addition, NS-1017 reduced the labelling of K80C (Figure 7A). In contrast, the labelling of other hydrophilic residues in TMD1, as well as of residues forming the

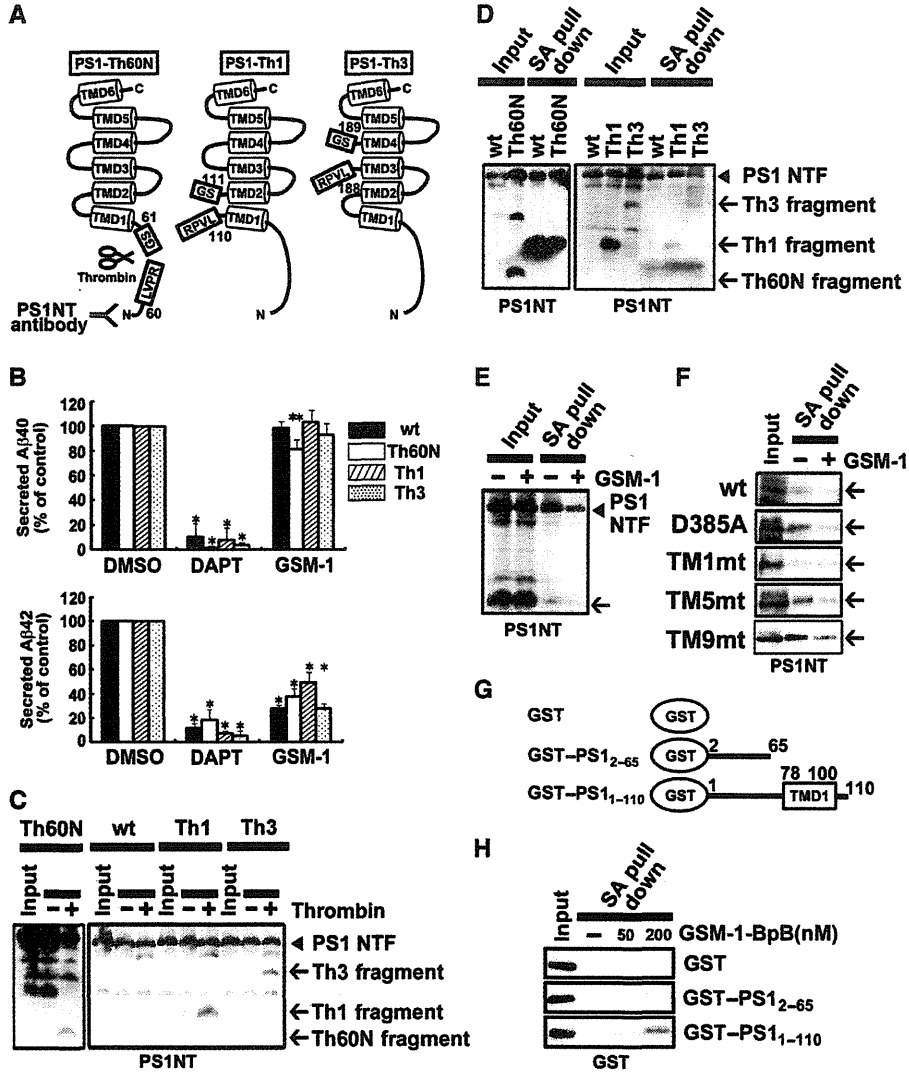


**Figure 6** Identification of TMD1 of PS1 as the binding site of GSM-1-BpB. (**A**) Schematic representation of the NTF of mutant PS1 inserted with a thrombin-cleavage sequence (squares) at different sites (PS1-Th60N, PS1-Th1 and PS1-Th3). (**B**) Effect of DAPT (10 μM) or GSM-1 (1 μM) on secreted Aβ from DKO cells stably expressing APPNL and PS1 mutants ($n=3$, mean ± s.d., $^*P<0.01$, $^{**}P<0.05$ at Student's *t*-test). (**C**) Thrombin digestion experiments of PS1-Th60N, PS1-Th1 and PS1-Th3. PS1 NTF and the cleaved fragments were indicated by an *arrow* and *arrowheads*. (**D**) Thrombin digestion experiments after PAL by GSM-1-BpB (1 μM). Note that cleaved Th1 or Th3 fragment, but not Th60N fragment, was precipitated and detected by anti-PS1 NTF antibody. (**E**) Preincubation by GSM-1 (200 μM) decreased the labelling of both PS1 NTF (*arrowhead*) and Th1 fragment (*arrow*). (**F**) PAL experiment of TM-swap mutant PS1 by GSM-1-BpB (1 μM). GSM-1-BpB labelled holoprotein forms of PS1 (*arrows*). Note that TM1mt PS1 was never labelled by GSM-1-BpB. (**G**) Schematic representation of recombinant proteins used in this study. (**H**) PAL experiment for recombinant proteins by GSM-1-BpB. Recombinant GST-PS1$_{1-110}$, but not GST or GST-PS1$_{2-65}$ (0.5 μg each), was labelled by GSM-1-BpB in a dose-dependent manner.

catalytic site in TMD6 (i.e., A246C and L250C; Sato C *et al*, 2006) was not altered by phenylpiperidine-type GSMs (Figure 7A and B). These data indicate that the water accessibility of the cytosolic side of TMD1 is specifically altered by the binding of phenylpiperidine-type GSMs. Taken altogether, we conclude that the phenylpiperidine-type GSMs directly and specifically target the TMD1 of PS1, leading to an alteration in the conformation of the catalytic pore structure of the γ-secretase, and thereby modulating the Aβ42 production (Figure 7C).

## Discussion

By a combination of chemical biology and molecular biology approaches, we have identified TMD1 of PS1 as the binding site of GSM-1, a representative and potent Aβ42-lowering GSM (Page *et al*, 2008). GSM-1 bound to PS1 either in an inactive, holoprotein form or within an enzymatically active complex, and induced conformational changes in the catalytic as well as the initial substrate binding sites in PS1. Thus, the mode of binding of GSM-1 to PS1 is different from that of the conventional GSIs, which selectively target the enzymatically active form of PS1 (Li *et al*, 2000; Morohashi *et al*, 2006; Fuwa *et al*, 2007; Watanabe *et al*, 2010). SCAM assay revealed that phenylpiperidine-type GSMs affected the water accessibility of Met84 located at the N-terminal region of TMD1 (Figure 7A). Notably, we have previously shown that Val82 and Leu85 in TMD1 of PS1 directly participate in the formation of the catalytic site of γ-secretase, while Met84

 

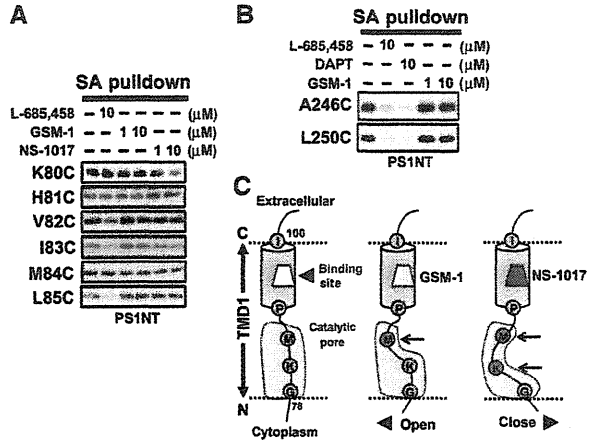


**Figure 7** Conformational changes in the cytoplasmic side of TMD1 of PS1 induced by phenylpiperidine-type γ-secretase modulators. (**A**, **B**) SCAM analysis of microsomes from DKO cells expressing single-Cys mt PS1 containing one Cys at the cytosolic side of TMD1 (**A**) or TMD6 (**B**) in the presence or absence of indicated compounds. All bands correspond to the biotinylated PS1 NTF. Note that the labelling of K80C or M84C was affected by preincubation with GSM-1 or NS-1017. (**C**) A hypothetical model of mode of action of phenylpiperidine-type GSMs. Membrane borders are indicated by dotted lines. N-terminal domain of TMD1 facing the catalytic pore is indicated by a blue region. C-terminal domain of TMD1 is shown by a pink cylinder. Yellow and red trapezoids indicate GSM-1 and NS-1017, respectively. The hypothetical open and close structure of the N-terminal domain of TMD1 of PS1 induced by GSMs is indicated.

does not, consistent with the notion that the phenylpiperidine-type GSMs affect the catalytic site in an allosteric manner. In addition to Met84, Aβ42-rasing NS-1017 decreased the water accessibility of Lys80, suggesting that a specific structural change around this region is linked to alteration in the net production of Aβ42 (Figure 7A and C). Interestingly, several FAD-linked mutations have been identified in the N-terminal region of TMD1 of PS1, suggesting that conformational changes in this region may be an important determinant for the Aβ42 generating activity. In contrast, analyses of the swap mutants revealed that the cytosolic side of TMD1 was dispensable for the binding of GSM-1. These data suggest that the phenylpiperidine-type GSMs directly target the hydrophobic C-terminal half of TMD1 (Figure 7C). Intriguingly, this hydrophobic half participates in the maintenance of the TMD-to-TMD interaction in PS1, which is involved in the assembly of the γ-secretase complex as well as the mechanism of substrate recognition (Takagi *et al*, 2010; Watanabe *et al*, 2010). Our data provide compelling evidence supporting the notion that phenylpiperidine-type GSMs modulate the γ-secretase activity by directly targeting the hydrophobic extracellular/luminal side of the PS1 TMD1, leading to a conformational change of the hydrophilic cytosolic side (Figure 7A and C).

Biochemical analyses indicate the presence of two product lines by γ-secretase to generate Aβ40/43 and Aβ38/42 from Aβ49 and Aβ48, respectively (Qi-Takahara *et al*, 2005; Takami *et al*, 2009). Here, we showed that only the Aβ38/42-generating activity was modulated by GSM-1 without affecting the levels of AICD as well as of other Aβ species including Aβ45, the latter being the possible cognate precursor for Aβ42. This is consistent with the previous findings that an NSAID-type GSM, sulindac sulphide, exclusively increased the release of tetrapeptide VVIA, which is derived from Aβ42 upon generation of Aβ38 (Takami *et al*, 2009). These results lead us to speculate that the Aβ42-lowering GSMs selectively alter the proteolytic activity upon the stepwise cleavage to generate Aβ38. The decrease in the binding of L-852,646 (a transition state analogue photoprobe) by GSM-1 reflects a structural change in the catalytic site. In contrast, Aβ40 generation was never affected by GSM-1. Notably, alignments of scissile bonds in the two product lines (i.e., Aβ49-46-43-40 and Aβ48-45-42-38) are located on distinct interfaces of the α-helical model of the substrate (Qi-Takahara *et al*, 2005; Takami *et al*, 2009). However, the result that GSM-1 decreased the labelling of PS1 NTF by pep.11-Bt may indicate an allosteric effect on the structure of the initial substrate binding site that determines Aβ42 generation. Thus, the selective modulation of Aβ38/42 generation suggests that GSM-1 affects both the catalytic pocket and the recognition mechanism of the helical interface during the processive cleavage of Aβ48/45/42.

A subset of NSAID-type GSMs have been reported to directly target the TMD of APP, especially the GXXXG motif (Kukar *et al*, 2008; Richter *et al*, 2010). However, this notion contradicts with the previous findings that several GSMs modulate the γ-secretase-mediated cleavage of substrates other than APP (i.e., Notch); these GSMs affect the cleavage of APP mutated at the GXXXG motif too (Okochi *et al*, 2006; Page *et al*, 2010). Moreover, the activity of SPP, a protease homologous to γ-secretase, also was affected by GSMs (Sato T *et al*, 2006). In this study, we demonstrated the direct biotinylation of PS1 NTF as well as of SPP by GSM-1-BpB, suggesting that the phenylpiperidine-type GSM bound to these enzymes. However, it remains possible that GSMs target the interface between the enzyme and the substrate upon modulation of the Aβ42 production. In this case, GSM might form a tripartite complex with the PS1 TMD1 and the substrate (Uemura *et al*, 2010). In fact, both the hydrophobic region of TMD1 of PS1 and the GXXXG motif in APP TMD are predicted to be located at similar topological position within the lipid bilayer: dual roles of TMD1 in the γ-secretase-mediated cleavage (i.e., substrate recognition as well as catalytic reaction) fit with this hypothesis (Takagi *et al*, 2010). However, we are not able to exclude the possibility that different domains of PS1 or other γ-secretase subunits are also involved in the modulation of Aβ42 generation, since PAL detects the closest target molecule located in proximity to the photoactivatable moiety. Intriguingly, we previously reported that the N-terminus of Pen-2, which is directly bound to TMD4 of PS1 (Watanabe *et al*, 2005), is involved in the selective modulation of Aβ42 production as well as the conformation of PS1 (Isoo *et al*, 2007; Uemura *et al*, 2009). And recently, a GSM with a phenylimidazole pharmacophore was reported to primarily target Pen-2 (Kounnas *et al*, 2010). In this regard, it is tempting to speculate that TMD1 and TMD4 of PS1, together with Pen-2, form the modulator binding site in the γ-secretase complex. It is also possible that different types of GSMs show distinct preference of interaction with γ-secretase components or substrates. Further detailed structural analysis (i.e., X-ray crystallography or NMR) of PS1, in a state coupled with a GSM and a substrate, would be required for the understanding of a variety in the molecular action of different GSMs.

In sum, we revealed the specific molecular action of the phenylpiperidine-type GSMs on PS1 TMD1 using a comprehensive strategy based on chemical biology. Such an approach would be useful for the rational design of small compounds in the development of effective therapeutics for AD.

## Materials and methods

### *Compounds, peptides and antibodies*

GSM-1, GSM-1-BpB, NS-1017 and GSM-1-amide-BpB were synthesized as described in Supplementary data. DAPT was synthesized as previously described (Morohashi *et al*, 2006). $(Z\text{-}LL)_2$-ketone, compound E, L-685,458, pep.11 and pep.11-Bt were purchased from Calbiochem, Bachem, Peptide Institute, Inc. and Ito Lifescience, respectively. CE-BpB3 (Fuwa *et al*, 2007) and L-852,646 (Li *et al*, 2000) were kindly provided from Drs H Fuwa (Tohoku University) and Y Li (Sloan-Kettering Cancer Center). The rabbit polyclonal antibodies anti-PS1 CTF (G1L3) and anti-Pen2 (PNT3) were raised as described (Tomita *et al*, 1999; Isoo *et al*, 2007). Anti-GST rabbit polyclonal antibody was purified using Glutathione sepharose 4B (GE Healthcare). Anti-PS1 NTF (PS1NT) and anti-SPP (SPPc) were kindly gifted from Drs G Thinakaran (University of Chicago) and T Golde (Universtiy of Florida). Anti-PS1-NTF 121121 (R&D Systems), anti-nicastrin N1660 (Sigma), anti-APP-CTF (Immuno-Biological Laboratories), anti-Aph-1aL O2C2 (Covance), anti-myc 9B11 (Cell Signaling Technology), anti-human Aβ 82E1 (Immuno-Biological Laboratories) and anti-biotin (Bethyl) were purchased from indicated vendors. The monoclonal antibody anti-α-tubulin AA4.3 developed by Dr C Walsh was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by The University of Iowa, Department of Biology, Iowa City, IA.

### *Plasmid construction and cell culture manipulation*

cDNAs encoding mutant PS1 were generated by long PCR-based QuikChange™ strategy (Stratagene). cDNAs encoding PS1, APP carrying Swedish mutation (APPNL) and NotchΔE (Kopan *et al*, 1996) were inserted into pMXs-puro (Kitamura *et al*, 2003). For construction of the thrombin-cleavable PS1 mutant, thrombin-cleavage sequence (CTGGTTCCGCGTGGATCC) was inserted into PS1 cDNA in pMXs-puro. To produce recombinant proteins, cDNAs encoding $PS1_{2-65}$ and $PS1_{1-110}$ were cloned into pGEX-6P-1 vector (GE Healthcare). Maintenance of cultured cells and infection of recombinant viruses were done as previously described (Hayashi *et al*, 2004; Watanabe *et al*, 2005, 2010; Morohashi *et al*, 2006; Ogura *et al*, 2006; Sato C *et al*, 2006; Fuwa *et al*, 2007; Takagi *et al*, 2010).

### *Cell-based, cell-free and in-vitro γ-secretase assay*

Immunoblot analysis was performed as previously described (Tomita *et al*, 1997, 1999). To monitor the cleavage of Notch, luciferase assay using HEK293 cells stably expressing APPNL/NotchΔE/EGFP/UAS-firefly luciferase was performed as previously described (Isoo *et al*, 2007; Imamura *et al*, 2009). For secreted Aβ levels, conditioned media from DKO cells co-expressing APPNL and PS1 mutant were analysed by two-site enzyme-linked immunosorbent assay (ELISA; Tomita *et al*, 1997) or immunoblotting using Urea/SDS–PAGE gel system as described (Qi-Takahara *et al*, 2005; Kakuda *et al*, 2006; Osawa *et al*, 2008). For cell-free γ-secretase assay, membranes of CHO cells stably expressing C99 were collected and analysed as described previously (Kakuda *et al*, 2006; Osawa *et al*, 2008). In all, 2.5 mg/ml microsomes in homogenized buffer (20 mM HEPES (pH 7.0), 140 mM KCl, 250 mM sucrose, 1 mM EGTA) containing 0.5 mM DIFP, 0.5 mM PMSF, 1 μg/ml TLCK, 1 μg/ml antipain, 1 μg/ml leupeptin, 10 μg/ml phosphoramidon, 1 mM EGTA, 5 mM EDTA were preincubated with various compounds on ice for 30 min. Following, microsomes were incubated at 37°C for 6 h. For detection of the γ-secretase activity in solubilized condition, 1% CHAPSO-solubilized membranes were co-incubated with APP-based recombinant substrate under 0.25% CHAPSO condition (Takahashi *et al*, 2003) and then analysed by immunoblot analysis. Recombinant γ-secretase complex as well as SPP was purified from baculovirus-infected Sf9 cells as previously described (Hayashi *et al*, 2004; Kakuda *et al*, 2006; Ogura *et al*, 2006; Fuwa *et al*, 2007). CHO cells expressing C99 and APP-based recombinant substrate were kindly provided from Drs S Funamoto and Y Ihara (Doshisha University). $IC_{50}$ values were calculated by plotting data on Kyplot software (Kyens Lab. Inc.).

### *PAL, SCAM and FLIM experiments*

Membranes from C57J/B6 mouse brain (3–5-month age) and cultured cells were homogenized with homogenize buffer and collected as described (Kakuda *et al*, 2006). PAL experiment utilizing avidin-biotin catch principle (Hofmann and Kiso, 1976) was performed as previously described (Morohashi *et al*, 2006; Fuwa *et al*, 2007; Imamura *et al*, 2009). For thrombin digestion after PAL, recentrifuged pellets were solubilized in thrombin digestion buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100). After incubation with 5 U/ml thrombin protease (GE Healthcare) at 37°C for 4 h, SDS was added to stop the enzymatic reaction. The biotinylated polypeptides were collected by streptavidin-Sepharose HP (GE Healthcare). For SCAM, all methanethiosulphonate reagents (Toronto Research Chemicals) were dissolved in dimethyl sulphoxide (DMSO) at 200 mM prior to use or stored at 80°C until use. The methods for SCAM and competition experiments using biotinylaminoethyl methanethiosulphonate have been described in detail before (Sato *et al*, 2006, 2008; Takagi *et al*, 2010). The proximity between the N-terminus and TM6-7 loop domain as an indicator of PS1 conformation was monitored by FLIM assay at the cell periphery of live cells expressing GFP-PS1-RFP construct as previously described (Uemura *et al*, 2009, 2010).

### *Supplementary data*

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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*Author contributions*: YO and TT designed the research. YO, SO and TT performed biochemical experiments. SY, TH, NS and TF synthesized the compounds. KU and OB performed the imaging experiments. YO, TT and TI wrote the paper.

## Conflict of interest

The authors declare that they have no conflict of interest.

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Neurobiology of Disease

# BACE1 Activity Is Modulated by Cell-Associated Sphingosine-1-Phosphate

**Nobumasa Takasugi,[1,3,4] Tomoki Sasaki,[1] Kunimichi Suzuki,[1] Satoko Osawa,[1] Hayato Isshiki,[1] Yukiko Hori,[1] Naoaki Shimada,[2] Takuya Higo,[2] Satoshi Yokoshima,[2] Tohru Fukuyama,[2] Virginia M.-Y. Lee,[5,6] John Q. Trojanowski,[5,6] Taisuke Tomita[1,4] and Takeshi Iwatsubo[1,3,4]**

Departments of [1]Neuropathology and Neuroscience and [2]Synthetic Natural Products Chemistry, Graduate School of Pharmaceutical Sciences, and [3]Department of Neuropathology, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan, [4]Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Bunkyo-ku, Tokyo 113-0033, Japan, and [5]Alzheimer's Disease Core Center and [6]Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

**Sphingosine kinase (SphK) 1 and 2 phosphorylate sphingosine to generate sphingosine-1-phosphate (S1P), a pluripotent lipophilic mediator implicated in a variety of cellular events. Here we show that the activity of β-site APP cleaving enzyme-1 (BACE1), the rate-limiting enzyme for amyloid-β peptide (Aβ) production, is modulated by S1P in mouse neurons. Treatment by SphK inhibitor, RNA interference knockdown of SphK, or overexpression of S1P degrading enzymes decreased BACE1 activity, which reduced Aβ production. S1P specifically bound to full-length BACE1 and increased its proteolytic activity, suggesting that cellular S1P directly modulates BACE1 activity. Notably, the relative activity of SphK2 was upregulated in the brains of patients with Alzheimer's disease. The unique modulatory effect of cellular S1P on BACE1 activity is a novel potential therapeutic target for Alzheimer's disease.**

## Introduction

Amyloid-β peptide (Aβ) is the major component of senile plaques deposited in the brains of patients with Alzheimer's disease (AD). Several lines of evidence suggest that the accumulation of Aβ is linked to the pathogenesis of AD (Tomita, 2009; De Strooper et al., 2010). Aβ is derived from amyloid-β precursor protein (APP) that is sequentially cleaved by two aspartate proteases, β- and γ-secretases. The major β-secretase is a type-1 transmembrane protein termed BACE1 (β-site APP cleaving enzyme 1) (Vassar et al., 2009). BACE1-deficient mice do not generate Aβ (Cai et al., 2001; Luo et al., 2001), but they exhibited hypomyelination (Hu et al., 2006; Willem et al., 2006) and altered neurological phenotype (Laird et al., 2005; Savonenko et al., 2008; Hu et al., 2010). However, modest reduction of BACE1 activity is sufficient for a significant reduction in brain Aβ deposition in AD model mice (McConlogue et al., 2007; Chow et al., 2010). Moreover, several reports indicate that the protein levels and/or the activity of BACE1 were increased in the brains of patients with sporadic AD (Fukumoto et al., 2002; Yang et al., 2003; Li et al., 2004; Ahmed et a., 2010), suggesting that subtle changes in BACE1 activity significantly impact on the pathomechanism of AD. BACE1 resides in the lipid raft, a membrane microdomain enriched in cholesterol and sphingolipids, and a significant role of lipids and microdomain is implicated in the regulation of the β-cleavage (Kalvodova et al., 2005; Rajendran et al., 2008; Vetrivel and Thinakaran, 2010). In this study, we focused on a biologically active lipid metabolite, sphingosine-1-phosphate (S1P). S1P functions as a ligand for G-protein-coupled receptor (GPCR)-type receptors from the extracellular side; alternatively, S1P has been shown to directly act on intracellular targets (Alvarez et al., 2007; Takabe et al., 2008; Pyne and Pyne, 2010). S1P is produced by phosphorylation of sphingosine by two related rate-limiting kinases, sphingosine kinase 1 (SphK1) and SphK2 (see Fig. 1*A*). Although SphK1 and SphK2 show different kinetic properties and tissue expression patterns (Blondeau et al., 2007; Spiegel and Milstien, 2007), both kinases are functionally redundant in the production of S1P *in vivo* (Mizugishi et al., 2005). Here, we show that modulation of SphK and S1P degrading enzymes alters the Aβ generation by regulating the β-cleavage via direct action of S1P on BACE1 protein. Furthermore, we found that SphK2 activity is increased in the brains of patients with sporadic AD. These data unveil a novel regulatory mechanism of BACE1 linked to S1P levels in neurons, supporting the view that SphK2/S1P is a novel potential therapeutic target for AD.

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Correspondence should be addressed to Dr. Taisuke Tomita, Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. E-mail: taisuke@mol.f.u-tokyo.ac.jp.

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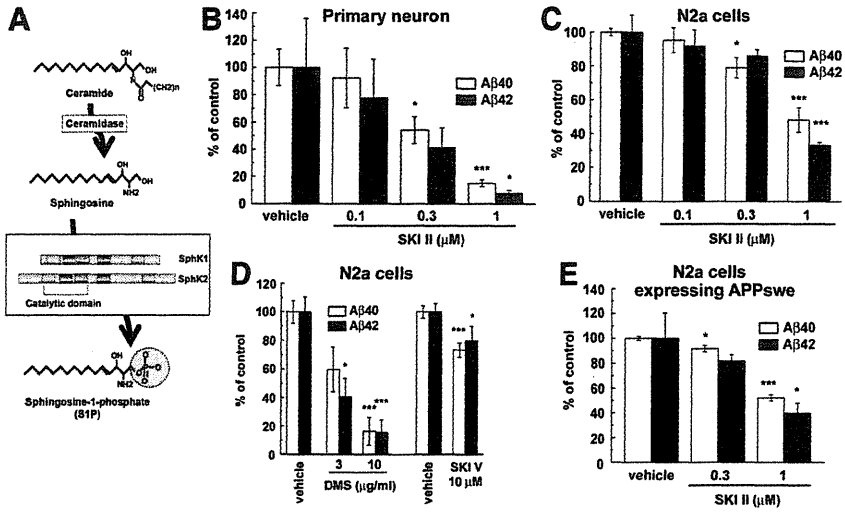


**Figure 1.** Sphingosine kinase inhibitors decreased the Aβ secretion from neuronal cells. ***A***, Schematic depiction of synthetic pathway for S1P. Five conserved regions of SphK are indicated by rectangles. Location of catalytic region is also indicated in this diagram. ***B–E***, Effects of SKI on secretion of $A\beta_{40}$ and $A\beta_{42}$ from neuronal cells. The levels of secreted Aβ in conditioned media were quantified by ELISAs. Mean ± SEM percentages of the relative ratio of secreted Aβ to levels in untreated control are indicated. $*p < 0.05$, $***p < 0.001$ by Student's *t* test. ***B***, Levels of secreted Aβ from mouse primary cortical neurons (7 d *in vitro*) after treatment with SKI II for 24 h ($n = 4$). ***C***, Levels of secreted Aβ from N2a cells after treatment with SKI II for 24 h ($n = 4$). ***D***, Levels of secreted Aβs from N2a cells after treatment with DMS or SKI V for 24 h ($n = 4–7$). ***E***, Levels of secreted Aβ measured by human Aβ-specific ELISA from N2a cells stably expressing Swedish mutant of APP after treatment with SKI II for 24 h ($n = 4$).

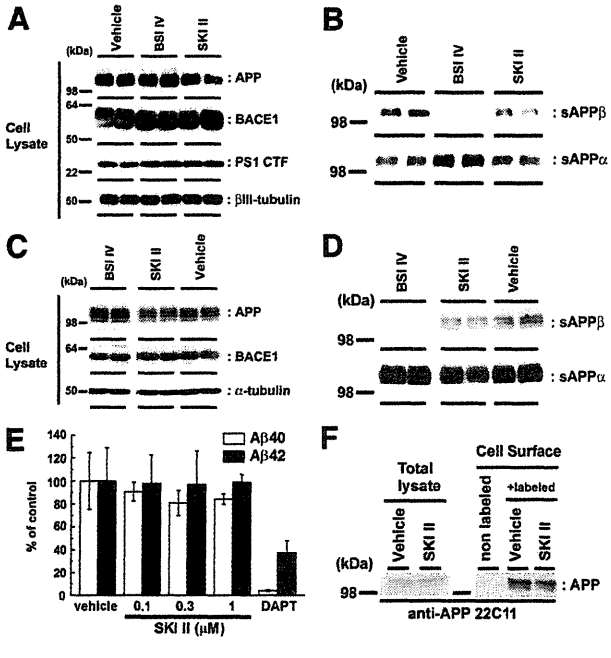


**Figure 2.** SKI II decreased the β-secretase cleavage products. ***A–D***, Immunoblot analyses of the protein levels of APP derivatives and BACE1 in neuronal cells. Immunoblot analysis of cell lysates (***A***) and cultured media (***B***) of mouse primary cortical neurons (7 d *in vitro*) treated with BACE inhibitor IV (BSI IV; 1 μM) or SKI II (1 μM) for 24 h in duplicate ($n = 3$; representative results are shown). Immunoblot analysis of cell lysates (***C***) and cultured media (***D***) of naive N2a cells treated with BACE inhibitor IV (BSI IV; 1 μM) or SKI II (3 μM) for 24 h. ***E***, Levels of secreted human Aβ from N2a cells overexpressing SC100 after treatment with SKI II for 24 h. Secreted human $A\beta_{40}$ and $A\beta_{42}$ were detected by human Aβ-specific ELISA ($n = 4$; mean ± SEM). ***F***, Effect of SKI II (1 μM) on the cell-surface levels of APP in naive N2a cells. After treatment with vehicle or SKI II for 24 h, N2a cells were biotinylated by sulfo-NHS-biotin and pulled down by streptavidin beads.

## Materials and Methods

*Compounds.* *N*-[*N*-(3,5-Difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester (DAPT) was synthesized as described previously (Kan et al., 2003). ABC294640 [3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide] was synthesized according to the reported procedure (U.S. Patent 2006287317). Sphingosine kinase inhibitor (SKI) II (Sigma-Aldrich), SKI V (Sigma-Aldrich), *N*,*N*-dimethylsphingosine (DMS) (Cayman Chemical), β-secretase inhibitor IV (Calbiochem), S1P (Calbiochem), and 2-acetyl-4-tetrahydroxybutylimidazole (THI) (Matreya) were purchased from the indicated vendors. DAPT, β-secretase inhibitor IV, SKI II, and SKI V were dissolved in DMSO, DMS were dissolved in ethanol, and S1P was dissolved in 3 mM NaOH. Lipid immobilized agarose beads were purchased from Echelon. Synthetic $A\beta_{1-42}$ peptides (Peptide Institute) were solubilized at a concentration of 0.6 mg/ml in PBS and incubated at 37°C for 24 h to form Aβ fibrils (Hori et al., 2007).

*Antibodies and immunological methods.* PS1 C-terminal fragment (CTF) (G1L3) was raised as described previously (Tomita et al., 1999). The following antibodies were purchased from the indicated vendors: human Aβ 82E1 (catalog #10323; Immuno-Biological Laboratories), APP (18) (catalog #28053; Immuno-Biological Laboratories), BACE1 (c) (catalog #18711; Immuno-Biological Laboratories), APP (c) (catalog #18961; Immuno-Biological Laboratories), anti-mouse/rat APP (597) (catalog #28055; Immuno-Biological Laboratories), anti-BACE1 (42) (catalog #28051; Immuno-Biological Laboratories), anti-sAPPβwt (catalog #18957; Immuno-Biological Laboratories), SphK2 (P-19) (SC-22704; Santa Cruz Biotechnology), TRAF2 (C-20) (SC-876; Santa Cruz Biotechnology), SphK1 (catalog #10006822; Cayman Chemical), α-Tubulin DM1A (T9026; Sigma-Aldrich), α-actin AC-40 (A4700; Sigma-Aldrich), anti-βIII-tubulin Tuj1 (MAB1195; R & D Systems), APP N-terminal 22C11 (MAB348; Millipore), and anti-Myc 9B11 (catalog #2276; Cell Signaling Technology). The samples were analyzed by immunoblotting or two-site ELISAs for the detection of Aβ as described previously (Iwatsubo et al., 1994; Tomita et al., 1997). For immunoblot detection for sAPPα and sAPPβ in cultured media, anti-human/mouse APP (597) and anti-sAPPβwt antibodies were used. Specificities of APP antibodies were shown previously (Fukumoto et al. 2010). For lipid binding assay, naive Neuro-2a (N2a) cell lysates or recombinant BACE1 [corresponding to its extracellular domain with a 10×His tag (catalog #931AS; R & D Systems)] were solubilized with 40 mM HEPES buffer, pH 7.4, containing 150 mM NaCl, 0.5% NP-40, and Complete protease inhibitor cocktail (Roche Applied Science) and spun down. Supernatants were coincubated with the lipid coated beads (Echelon Biosciences) or Nickel-NTA beads (Qiagen) that were preincubated with 3% BSA in HEPES buffer. After 3 h, bound proteins were eluted by sample buffer and subjected to immunoblotting.

*Cell culture and transfection.* Expression constructs for human APP carrying Swedish mutation (APPNL), SC100, and NΔE were described previously (Kopan et al., 1996; Tomita et al., 1997). cDNAs encoding SphK2, S1P phosphatase (SGPP1), and S1P lyase (SGPL1) were inserted into pcDNA3.1DV5–His/TOPO (Invitrogen) and mutant cDNAs were generated by long PCR-based Quikchange strategy (Stratagene). All constructs were sequenced using Thermosequenase (GE Healthcare) on an automated Sequencer (Li-Cor). N2a cells are maintained as described (Tomita et al., 1997). Primary cortical neurons were prepared from BALB/c mice at embryonic day 16 and grown in Neurobasal medium supplemented with B27 (Invitrogen) for 7 d (Fukumoto et al., 1999). Plasmid transfection was performed using Lipofectamine2000 (Invitro-

gen) or Fugene 6 (Roche Applied Science) following the instructions of the manufacturer. Small interfering RNA (siRNA) duplexes targeting to control, mouse *Sphk1* and *Sphk2* (target sequences: *Sphk1*, 5′-CTG GAC CAG TTG CAT ATA GAA-3′; *Sphk2*, 5′-TAG GCC TGG CCT CGT TGC ATA-3′) were purchased from Qiagen. Each siRNA was reversely transfected in N2a cells using LipofectAMINE RNAiMax (Invitrogen) following the instructions of the manufacturer.

In vitro *secretase activity assay.* For *in vitro* β-secretase assay, recombinant human BACE1 (catalog #931AS; R & D Systems) or cell membranes of N2a, primary neuronal cells, or mouse brain (Hashimoto et al., 2002; Takasugi et al., 2003) were used as enzyme sources. After homogenization in 10 mM Tris, pH 7.0, the enzyme fractions were acidified by 25 mM $CH_3COONa$, pH 4.5, and incubated with the β-secretase-specific substrate JMV2236 (Bachem) at 37°C at the indicated times. Fluorescence of the fractions was measured at 320 and 420/430 nm as excitation and emission wavelengths, respectively. *In vitro* α-secretase assay was performed using SensoLyte 520 TACE (α-Secretase) Activity Assay kit (Anaspec) following the instructions of the manufacturer. N2a cells were treated with the indicated reagent for 24 h and collected cell membrane. Ten micrograms of protein were used as enzymatic source, and reaction were performed for 30 min.

In vitro *SphK2 activity assay.* Specific SphK2 activity assay was performed according to a previous report (Zemann et al., 2006; Don et al., 2007). After 48 h incubation, cells were washed with iced PBS and lysed by freeze–thaw cycle in 50 mM HEPES, pH 7.4, 10 mM KCl, 15 mM $MgCl_2$, 0.1% Triton X-100, 20% glycerol, 2 mM orthovanadate, 2 mM dithiothreitol, 10 mM NaF, 1 mM deoxypyridoxine, and EDTA-free complete protease inhibitor (Roche Applied Science). Lysates were cleared by centrifugation at 15,000 rpm for 5 min. The lysates and NBD-Sphingosine (10 μM final; Avanti Polar Lipids) were mixed in the reaction buffer (50 mM HEPES, pH 7.4, 15 mM $MgCl_2$, 0.5 mM KCl, 10% glycerol, and 2 mM ATP) and incubated for 30 min at 30°C. The reactions were stopped by the addition of equal amount of 1 M potassium phosphate, pH 8.5, followed by addition of 2.5-fold chloroform/methanol (2:1), and then centrifuged at 15,000 rpm for 1 min. Only the reactant NBD-S1P, but not the substrate NBD-Sphingosine, was collected in alkaline aqueous phase. After aqueous phase was combined with an equal amount of dimethylformamide, the fluorescence value was read. For the analysis of human brains, Tris-soluble fractions were used as an enzyme source. Specificity of this method has been described previously (Zemann et al., 2006).

*SKI II treatment in wild-type and AD model mice.* All experiments using animals in this study were performed according to the guidelines provided by the Institutional Animal Care Committee of the Graduate School of Pharmaceutical Sciences, The University of Tokyo. All animals were maintained on food and water with a 12 h light/dark cycle. Wild-type female mice (C57BL; SLC Japan) at 8 weeks of age were used. SKI II was dissolved at 2 μM in 40% DMSO/PBS. Each 2 μl solution was administered by stereotaxic injection into the hippocampus (bregma −2.6 mm, 3.1 mm lateral, 2.4 mm depth). After 8 h, the hippocampus of injected and uninjected site were isolated. Hippocampus samples were solubilized with 10 mM Tris buffer containing 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and subjected to the sandwich ELISA for Aβ (Wako Chemical). A7 transgenic mice overexpress human APP695 harboring K670N, M671L, and T714I FAD mutations in neurons under the control of Thy1.2 promoter (Yamada et al., 2009). Female A7 mice at 6 months of age were used for subchronic treatment of SKI II. SKI II was dissolved in corn oil and injected orally for 7 d (50 $mg \cdot kg^{-1} \cdot d^{-1}$).

*Human brain samples.* Human brain samples from AD and aged control patients were derived from tissue bank at the University of Pennsylvania Alzheimer's Disease Core Center (ADCC) and the Center for Neurodegenerative Disease Research (CNDR). Control and AD brains were diagnosed symptomatically and pathologically at ADCC–CNDR as described (Arnold et al., 2010). All samples used for experimental measures were derived from frontal cortex under approval by the institutional review board, ADCC–CNDR, and institutional ethical committee of Graduate School of Pharmaceutical Sciences, The University of Tokyo. Brain samples were homogenized in TSI buffer (50 mM Tris HCl, pH 7.6, 150 mM NaCl, 0.5 mM diisopropyl fluorophosphate, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, 1 mg/ml antipain, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mg/ml Na-*p*-tosyl-L-lysine chloromethyl ketone) and centrifuged at 260,000 × *g* for 20 min. Supernatant was collected as Tris-soluble fraction and used for SphK assay.

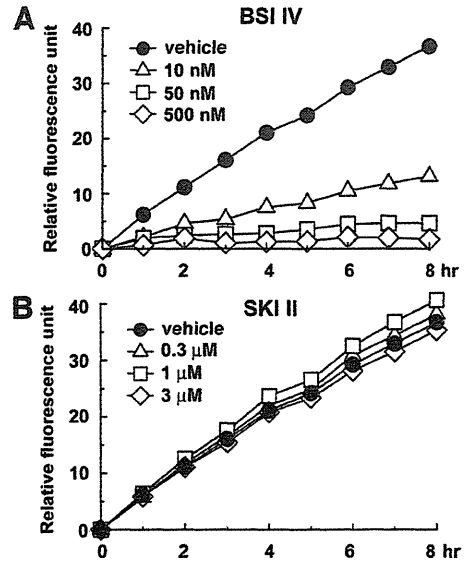


**Figure 3.** Effect of SKI II on the catalytic activity of BACE1. *In vitro* BACE1 activity assay using a fluorogenic BACE1-specific substrate. BACE inhibitor IV (BSI IV; ***A***) or SKI II (***B***) was coincubated with recombinant soluble BACE1 protein at indicated duration and concentrations. Relative fluorescence units were shown ($n = 3$).

## Results

### SphK inhibitors decreased Aβ secretion by reducing the β-cleavage of APP

To investigate the relationship between S1P and Aβ production, we focused on the activity of SphKs (Fig. 1*A*). Recently, several small compounds that specifically inhibit SphKs have been developed as anti-cancer drugs (Pyne and Pyne, 2010). Treatment with a SphK-selective inhibitor, SKI II (French et al., 2003, 2006), decreased the secretion of endogenous Aβ from mouse primary cortical neurons (Fig. 1*B*), as well as in mouse neuroblastoma N2a cells, in a dose-dependent manner (Fig. 1*C*). Two additional selective SphK inhibitors with different chemical structure (i.e., *N*,*N*-dimethylsphingosine and SKI V) also decreased the Aβ secretion from N2a cells (Fig. 1*D*). SKI II treatment decreased the Aβ secretion from N2a cells overexpressing the Swedish mutant form of human APP (APPNL) (Fig. 1*E*). These data suggest an inhibitory effect of SphK inhibitors on Aβ secretion. Notably, both $Aβ_{40}$ and $Aβ_{42}$ levels were affected in a similar manner in all following experiments. Next we analyzed the APP metabolism in SKI II-treated cells. SKI II treatment did not affect the expression levels of either BACE1 or presenilin-1, which is the γ-secretase catalytic component, in primary cortical neuron (Fig. 2*A*) or N2a cells (Fig. 2*B*), respectively. Because the treatment of authentic BACE1 inhibitor IV abolished the secretion of β-secretase-mediated cleavage product of APP, i.e., sAPPβ, in conditioned media, SKI II treatment also caused a moderate but significant decrease in sAPPβ in primary cortical neurons (Fig. 2*C*) or N2a cells (Fig. 2*D*). In contrast, SKI II showed no effect on the cleavage of the C-terminal stub of human APP (SC100) that serves as a direct substrate of γ-secretase (Fig. 2*E*). Moreover, SKI II treatment caused neither an increase in *in vitro* α-secretase activity in cell membranes (78.9 ± 2.3% compared with DMSO treatment, $n = 4$) nor change in the level of APP on the plasma membrane (Fig. 2*F*). Collectively, these data strongly suggest that SKI II directly affected the β-cleavage of APP.

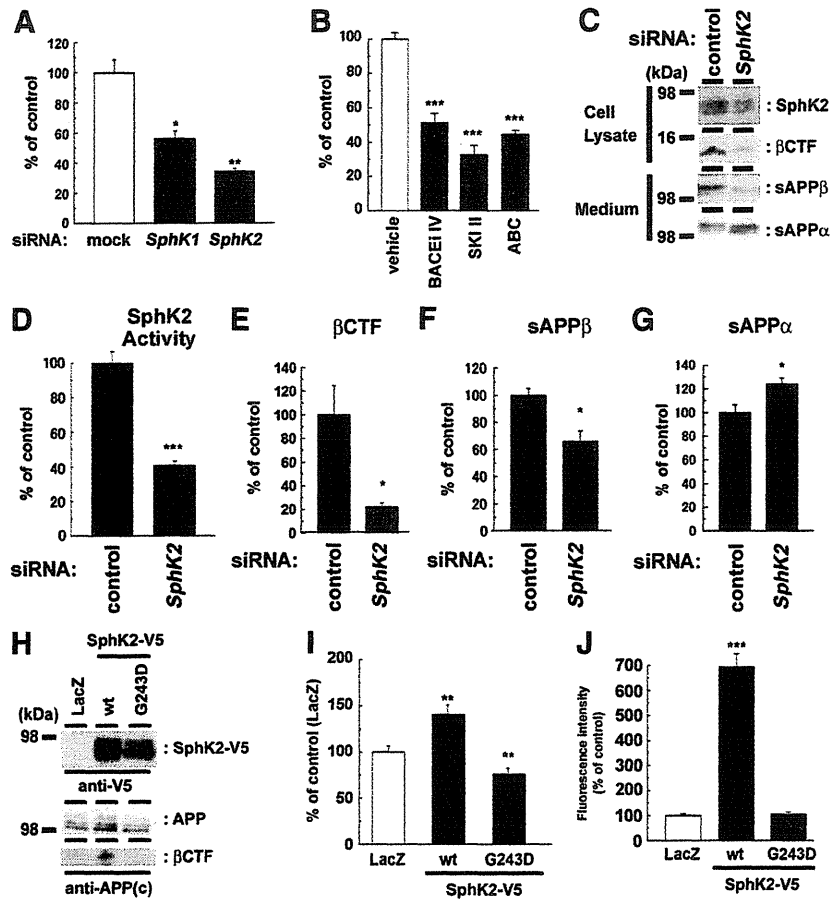


**Figure 4.** SphK2 activity modulated the β-secretase cleavage products. ***A***, N2a cells were transiently transfected with siRNAs against endogenous SphKs. After 48 h transfection, media were replaced and further incubated for 24 h. Levels of secreted Aβ were quantified by ELISA ($n = 3$; mean ± SEM; $^*p < 0.05$, $^{**}p < 0.01$). ***B***, Levels of secreted Aβ from N2a cells treated with BACE inhibitor IV (BACEi IV), SKI II, or SphK2-selective inhibitor ABC294640 (ABC) for 24 h ($n = 3$; mean ± SEM; $^{***}p < 0.001$). ***C–G***, Effect of transient *SphK2* knockdown on APP derivatives in N2a cells. Representative immunoblot analysis was shown in ***C***. *In vitro* SphK2 activity (***D***) as well as βCTF (***E***) in cell lysates and the amount of sAPPβ in conditioned media (***F***) were significantly decreased by knockdown of *SphK2*. In contrast, the level of sAPPα in conditioned media (***G***) was significantly increased (quantitated by densitometric analysis; $n = 4$; mean ± SEM; $^*p < 0.05$). ***H–J***, Effect of SphK2 on N2a cells coexpressing Swedish mutant of APP. wt, Wild type. Representative immunoblot analysis was shown in ***H***. Overexpression of SphK2, but not inactive mutant (G243D), increased the levels of Aβ production (***I***) as well as SphK2 activity *in vitro* (***J***) ($n = 4$; mean ± SEM; $^*p < 0.05$, $^{**}p < 0.01$).

## S1P metabolism coordinately modulates the β-cleavage of APP

To test whether SKI II directly inhibited the enzymatic activity of BACE1, a major β-secretase in neurons, we coincubated SKI II in *in vitro* assay using recombinant soluble BACE1 corresponding to its extracellular domain. However, SKI II itself did not affect the catalytic activity of recombinant BACE1 (Fig. 3). This result suggests that SKI II modulates the β-cleavage through the inhibition of SphK. Consistently, RNA interference (RNAi) against either SphK1 or 2 significantly decreased the Aβ production in N2a cells (Fig. 4*A*). Notably, knockdown of SphK2 showed a potent inhibitory effect. Supporting this result, SphK2-selective inhibitor ABC294640 (French et al., 2010) inhibited the Aβ generation in N2a cells similarly to that by SKI II (Fig. 4*B*), indicating that SphK2 plays a major role in the β-cleavage modulation. Thus, we focused on SphK2 in the following part of the study. RNAi against SphK2 resulted in a significant decrease in the levels of SphK2 expression (Fig. 4*C*), kinase activity (Fig. 4*D*), βCTF (Fig. 4*E*), as well as the secretion of sAPPβ (Fig. 4*F*), whereas the levels of secreted sAPPα was increased (Fig. 4*G*). In contrast, overexpression of SphK2, but not of an inactive mutant (G243D), significantly increased the levels of βCTF and secreted Aβ (Fig. 4*H,I*), along with an augmentation in SphK2 activity *in vitro* (Fig. 4*J*). Notably, coexpression of inactive SphK2 mutant decreased the levels of secreted Aβ (Fig. 4*I*), indicating that this mutant functions in a dominant-negative manner (Yoshimoto et al., 2003). These data indicate that cellular SphK2 activity is tightly correlated with the β-cleavage of APP.

To further test whether S1P, which is produced by SphK activity, is the regulator of β-cleavage, we examined the effects of S1P degrading enzymes on APP processing. SGPP1 dephosphorylates S1P to sphingosine, and SGPL1 irreversibly cleaves S1P to generate phosphoethanolamine and a long-chain aldehyde (Fig. 5*A*) (Alvarez et al., 2007; Takabe et al., 2008). Thus, these enzymes decrease the cellular S1P levels with different end products. Overexpression of either SGPP1 or SGPL1 in N2a cells strongly reduced the levels of βCTF and secreted Aβ (Fig. 5*B–D*). In contrast, the expression of catalytically inactive SGPL1 harboring K353L mutation (Reiss et al., 2004) showed no effect. Moreover, the inhibition of SGPL1 by THI (Schwab et al., 2005) caused a significant increase in Aβ secretion from mouse primary neurons (Fig. 5*E*), suggesting that the enzymatic activity of S1P degrading enzymes is important for the modulation of β-cleavage. Together, these data indicate that the S1P metabolism coordinately modulates the β-cleavage of APP.

## Cell-associated S1P directly modulates BACE1 activity

A proportion of newly synthesized S1P is secreted, whereas others remain associated with cells. In general, extracellular S1P poorly permeates into the cells (Kihara et al., 2003) and functions as a ligand for cell-surface GPCR-type receptors (Alvarez et al., 2007; Takabe et al., 2008). However, extracellular application of S1P failed to restore the reduced Aβ secretion by SKI II treatment or SphK2 knockdown (Fig. 6*A,B*), suggesting that cell-associated S1P is involved in the regulation of β-cleavage. Next we tested the effect of S1P on the intrinsic activity of membrane-bound BACE1 in a cell-free assay. Both pretreatment of SKI II (Fig. 6*C*) and SphK2 knockdown (Fig. 6*D*) on N2a cells significantly decreased the BACE1 activity in the membrane fractions *in vitro*, implicating that the levels of S1P within cells correlate with BACE1 activity. Supporting this notion, addition of S1P into the microsome fraction significantly increased the intrinsic BACE1 activity (Fig. 6*E*). These data implicate the direct action of S1P on BACE1 activity rather than the cell-surface receptor-mediated modulation. To provide additional evidence that S1P directly modulates BACE1, we examined binding of endogenous BACE1 in N2a cell lysates to S1P immobilized on agarose beads (Fig. 6*F*). We confirmed the specific binding of

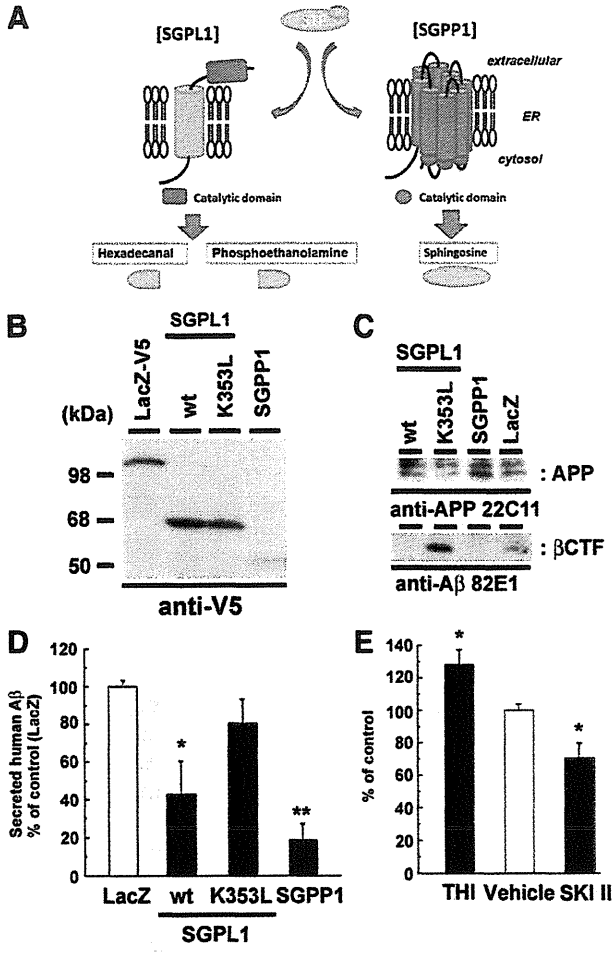


**Figure 5.** Effect of S1P degrading enzymes on Aβ production. ***A***, Schematic view of S1P degradation pathway. Note that SGPL1 and SGPP1 generate different degradation products of S1P. ER, Endoplasmic reticulum. ***B–D***, Effects of the overexpression of V5-tagged S1P degrading enzymes on APP metabolism in N2a cells. N2a cells were cotransfected with S1P degrading enzymes and Swedish mutant of APP. After 24 h transfection, media were replaced and further incubated for 24 h. Immunoblot analysis of S1P degrading enzymes (***B***) and APP derivatives (***C***) are shown. Human APP-derived βCTF was specifically detected by an anti-human Aβ N-terminus antibody (82E1). wt, Wild type. ***D***, The levels of secreted human Aβ was detected by human Aβ-specific ELISA ($n = 4$; mean ± SEM; $*p < 0.05$, $**p < 0.01$). Note that overexpression of SGPL1 or SGPP1, but not SGPL1 carrying catalytically inactive mutation (K353L), decreased the generation of βCTF and the Aβ secretion from N2a cells. ***E***, The levels of secreted Aβ from mouse primary cortical neurons (7 d *in vitro*) treated with SGPL1 inhibitor THI (50 μg/ml) or SKI II (1 μM) for 24 h ($n = 4$; mean ± SEM; $*p < 0.05$).

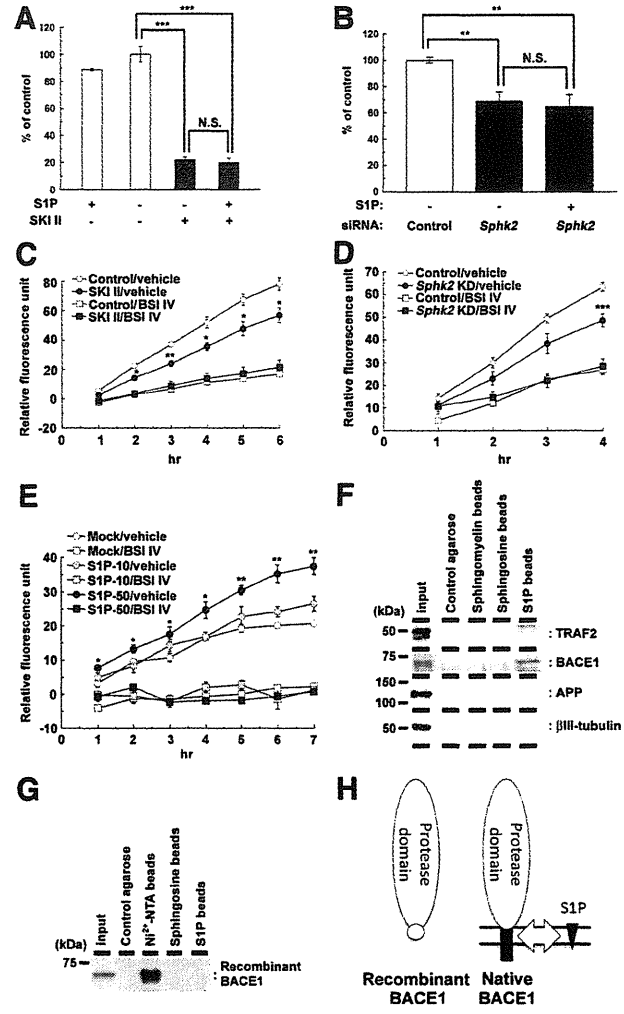


**Figure 6.** SKI II treatment decreased the β-secretase activity in cellular membrane. ***A***, ***B***, Effect of extracellularly added S1P (10 μM) on levels of secreted Aβ from mouse primary cortical neurons (7 d *in vitro*) after treatment with SKI II (1 μM) for 24 h (***A***) ($n = 4$; mean ± SEM) or from N2a cells after 48 h *SphK2* knockdown (***B***) ($n = 3$; mean ± SEM; $**p < 0.01$, $***p < 0.001$; N.S., no significant difference). Note that S1P failed to rescue the decrease in Aβ production either by SKI II or *SphK2* knockdown. ***C***, β-Secretase activity in the membrane fractions of N2a cells treated with vehicle or SKI II (1 μM) for 24 h. BACE inhibitor IV (BSI IV; 1 μM) was added to the *in vitro* assay ($n = 3$; mean ± SEM; $*p < 0.05$, $**p < 0.01$ vs control/vehicle). ***D***, β-Secretase activity in the membrane fractions of *SphK2* knockdown N2a cells. BACE inhibitor IV (BSI IV; 1 μM) was added to the *in vitro* assay ($n = 3$; mean ± SEM; $***p < 0.001$ vs control/vehicle). ***E***, Effect of S1P on β-secretase activity in the membrane fractions of mouse brain. S1P (10 or 50 μM) and BACE inhibitor IV (BSI IV; 1 μM) were added to the *in vitro* assay ($n = 3$; mean ± SEM; $*p < 0.05$, $**p < 0.01$ vs mock/vehicle). ***F***, ***G***, Association of BACE1 holoprotein with immobilized S1P. N2a cell lysates (***F***) or recombinant BACE1 with 10×His tag that lacks the transmembrane and cytoplasmic domains (***G***) were incubated with control agarose (no lipid), Nickel-NTA agarose, sphingomyelin, sphingosine, or S1P-coated affinity matrices (as indicated), and bound proteins were analyzed by immunoblotting. ***H***, Schematic model of the binding of BACE1 and S1P. S1P (black triangles) interacts with the C-terminal region of BACE1 (black squares), including the transmembrane domain, but not with the extracellular protease domain (white ovals). Location of 10×His tag is indicated by a white circle.

TRAF2 to S1P beads as described recently (Alvarez et al., 2010). Furthermore, endogenous BACE1, but not APP, was specifically pulled down by matrices carrying S1P. In contrast, recombinant BACE1 protein that lacks the transmembrane and cytoplasmic domains was never bound to S1P beads (Fig. 6*G*). Collectively, these results strongly suggest that the cell-associated S1P modulates the proteolytic activity of membrane-bound form of BACE1 via direct interaction (Fig. 6*H*).

### Roles of SphK2 activity in Alzheimer's disease

Data shown above indicate that the intracellular S1P is one of the important determinants for BACE1 activity. We further examined the impact of reduced S1P levels on Aβ levels *in vivo*. Stereotaxic injection of SKI II into wild-type mouse brain significantly decreased the amount of endogenous Aβ at hippocampus (Fig. 7*A*). Because SKI II exhibited a favorable bioavailability (French et al., 2006), we next orally administered SKI II to APP transgenic mice A7 overexpressing human APP carrying Swedish and Austrian mutations (Yamada et al., 2009). After 6 d treatment with SKI II in 6-month-old female mice, in which pathologically detectable amyloid plaques have not been developed, the total Aβ levels in brains were significantly decreased (Fig. 7*B*). These data indicate that the inhibition of SphK activity in APP

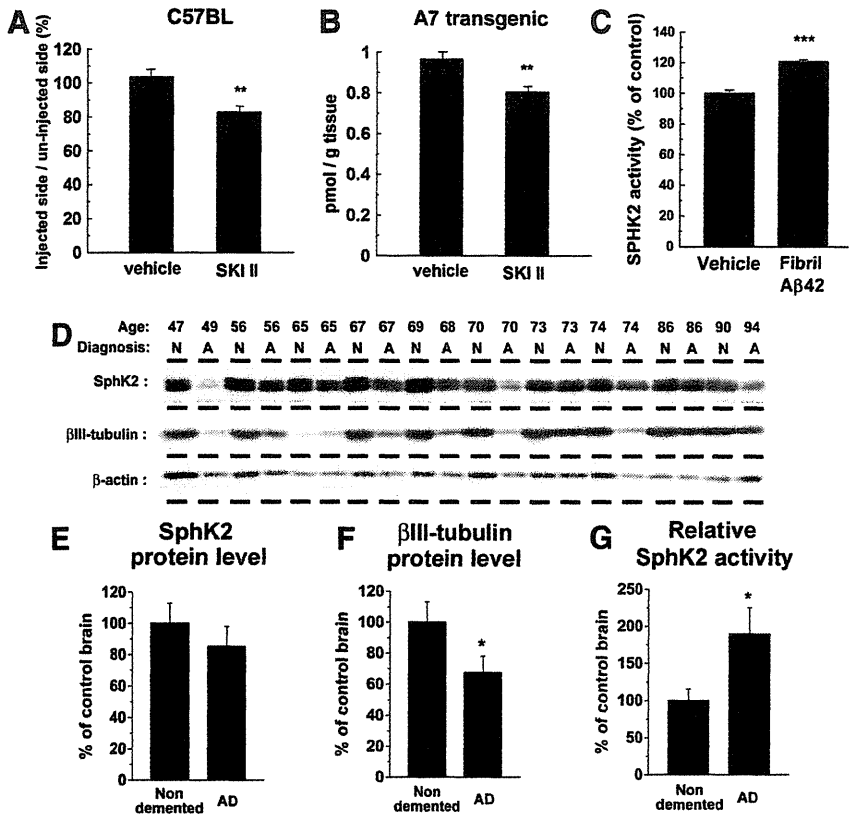


**Figure 7.** Role of SphK2 activity in AD brains. ***A***, Effect of the direct injection of SKI II into the hippocampus of nontransgenic wild-type female mice (C57BL) at 8 weeks of age. The levels of Tris-soluble Aβ in the injected side of hippocampus were divided by those in the uninjected side. Data represent relative ratio of each group ($n = 4$; mean ± SEM; $^{**}p < 0.01$). ***B***, Levels of Tris-soluble Aβ in the cerebral cortices of female A7 mice at 6 months of age after a 7 d treatment with SKI II (50 mg · kg$^{-1}$ · d$^{-1}$, p.o.). Total brain Aβ levels were measured by human-specific sandwich ELISA ($n = 5$; mean ± SEM; $^{**}p < 0.01$). ***C***, Effect of Aβ fibril on SphK2 activity in N2a cells. N2a cells were treated with $A\beta_{42}$ fibril (30 μM) overnight, and cell lysates were subjected to an *in vitro* SphK2 activity assay ($n = 3$; mean ± SEM; $^{***}p < 0.001$). ***D–F***, Immunoblot analysis of Tris-soluble fractions (15 μg of protein in each lane) from cortices of AD (denoted as A) or non-demented (denoted as N) individuals. Average protein levels of SphK2 (***E***) and βIII-tubulin (***F***) in each individual were analyzed by densitometric analyses ($^{*}p < 0.05$). ***G***, Average of *in vitro* SphK2 enzymatic activity of Tris-soluble fractions from brains of AD and non-demented individuals. The enzymatic activities of SphK2 were normalized by the protein levels of SphK2 quantified in ***D***.

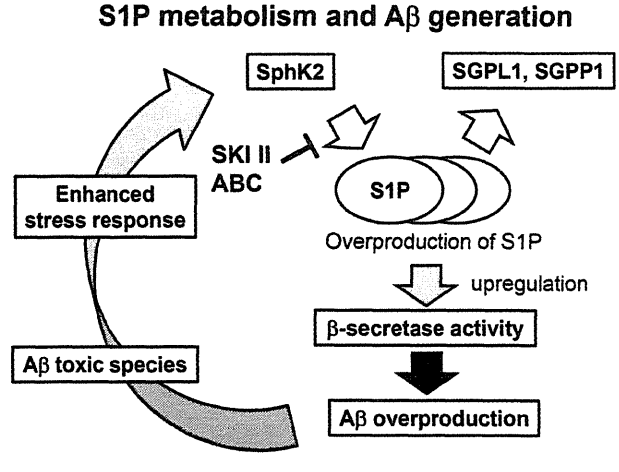


**Figure 8.** Schematic representation of the role of S1P metabolism in AD.

transgenic mouse brains has beneficial effects against Aβ production.

SphK activity is regulated by various stimuli and stress (Spiegel and Milstien, 2007). Intriguingly, treatment of N2a cells with fibrillized $A\beta_{42}$ caused a significant increase in the SphK2 activity (Fig. 7*C*), raising the possibility that the Aβ deposits in AD brains in turn augment Aβ production through SphK-mediated BACE1 activation in neurons. To further clarify the significance of SphK2 activity in the pathogenesis of sporadic AD, we compared the protein levels and the activities of SphK2 in cerebral cortices between sporadic AD patients and non-demented individuals (Fig. 7*D*). In contrast to the levels of α-actin, the levels of neuronal βIII-tubulin were significantly decreased in AD brains because of neuronal loss (Hempen and Brion, 1996). The protein levels of SphK2 also showed a decreased trend in AD brains (Fig. 7*E,F*), in accord with the previous description that the major SphK2-expressing cells are neurons (Blondeau et al., 2007). However, the relative *in vitro* activities of SphK2 were significantly upregulated in AD brains (Fig. 7*G*). These results provide compelling evidence that changes in the levels of cell-associated S1P in neurons, which is increased by a variety of stimuli including Aβ fibrils, modulate the proteolytic activity of BACE1, thereby forming a vicious cycle in the etiology of AD (Fig. 8).

## Discussion

Here we show that lowering the activity of SphK or increasing that of S1P degrading enzymes decreased the Aβ production by the inhibition of BACE1 activity *in vitro* and *in vivo*. Notably, SphK2 activity was increased by exposing cells to Aβ fibrils, and it also was increased in the postmortem cerebral cortices of AD patients. These results suggest that SphK2 and S1P are involved in the etiology of AD and novel potential therapeutic targets for AD.

Metabolites of sphingolipids are functionally interrelated with each other. Inhibition of SphK activity diminishes the generation of S1P and simultaneously increases the cellular levels of sphingosine and ceramide (Spiegel and Milstien, 2007; Pyne and Pyne, 2010). However, overexpression of SGPP1 or SGPL1, which decreases S1P levels by dephosphorylation or irreversible degradation, also decreased the levels of Aβ in N2a cells (Fig. 5). Moreover, direct addition of S1P to the membrane, but not on living cells, increased the β-secretase activity, suggesting that the cell-associated form of S1P per se plays a critical role for the modulation of BACE1 activity in neurons. Importantly, we observed a specific interaction of S1P with BACE1 holoprotein (Fig. 6*F*), in which transmembrane and intracellular domains of BACE1 are required (Fig. 6*G*). Thus, we hypothesize that S1P binds to BACE1 transmembrane/intracellular domain (Fig. 6*H*) and affects the proteolytic activity by altering the conformation or substrate accessibility. In good accordance with this, recent findings implicate intracellular S1P as a novel modulator for enzymes; S1P specifically binds to the histone deacetylases HDAC1 and HDAC2 and inhibited their enzymatic activity (Hait et al., 2009). Moreover, S1P targets to TRAF2 at the RING domain to

stimulate E3 ligase activity (Alvarez et al., 2010). It has been shown that functions of membrane-embedded as well as membrane-associated proteins are modulated by direct interaction with sphingolipids [e.g., activation of TrkA receptor by GM1 (Mutoh et al., 1995), inhibition of epidermal growth factor receptor by GM3 (Kawashima et al., 2009), activation of synaptobrevin (Darios et al., 2009), and functional modulation of stargazin by sphingosine (Sumioka et al., 2010)]. In these cases, lipid interactions are predicted to affect the conformation of functionally active domains located at the luminal or cytoplasmic sides. Moreover, it was shown previously that RTN3 inhibits the BACE1 activity via interaction with the transmembrane domain of BACE1 (He et al., 2004; Murayama et al., 2006). In addition, we have recently identified that a lipophilic, noncompetitive BACE1 inhibitor, TAK-070, directly targets the transmembrane domain of BACE1 (Fukumoto et al., 2010). These results collectively support the notion that targeting the transmembrane domain of BACE1, which harbors an allosteric modulatory function on the catalytic domain, might be a novel approach for the inhibition of the β-cleavage. Additional detailed analysis of molecular effects of S1P on BACE1 should be performed.

Several reports indicate that intrinsic activity of BACE1 is increased in AD brains (Fukumoto et al., 2002; Yang et al., 2003; Li et al., 2004; Ahmed et al., 2010), although the underlying molecular mechanism is essentially unknown. In this study, we found that treatment of cultured cells with Aβ fibrils augmented SphK2 activity, which was increased in AD brains, as well. Notably, intrinsic SphK2 activity is modulated by extracellular signal-regulated kinase (ERK) and fyn kinase (Olivera et al., 2006; Hait et al., 2007), which have been implicated in Aβ-mediated neurotoxicity (Crews and Masliah, 2010), suggesting the possibility that upregulation of SphK2 activity was mediated by aberrant phosphorylation by ERK and/or fyn kinase activity. Additional analysis would be required to understand the molecular connection between Aβ and SphK2 activity. Moreover, SphK2 activity was upregulated by neuronal stress, such as ischemia (Blondeau et al., 2007), which is also correlated with modulation of BACE1 activity (Wen et al., 2004; Tesco et al., 2007). Nevertheless, the increased SphK2 activity by Aβ fibril in neurons thereby may form a vicious cycle in the pathophysiology of AD (Fig. 8). Finally, SKI II treatment decreased the brain Aβ levels in APP transgenic mice, supporting the feasibility of SphK inhibition as a potential AD therapy. Especially, SphK2 single knock-out mice did not show significant developmental defects (Mizugishi et al., 2005). Moreover, SphK2 has been implicated in proapoptotic function, whereas SphK1 harbors anti-apoptotic effects (Liu et al., 2003; Maceyka et al., 2005). Thus, SphK2 selective inhibitors, e.g., ABC294640 (French et al., 2010), may be tolerable and suitable therapeutic agents for AD therapeutics. In conclusion, SphK2/S1P in brain might be a novel molecular target for AD therapeutics, and additional analysis for the regulatory mechanisms of β-secretase activity by SphK/S1P will facilitate the understanding of the pathogenesis of sporadic AD.

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