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

Yu Ohki¹, Takuya Higo², Kengo Uemura³,
Naoaki Shimada², Satoko Osawa¹,
Oksana Berezovska³, Satoshi Yokoshima²,
Tohru Fukuyama², Taisuke Tomita^{1,4,*}
and Takeshi Iwatsubo^{1,4,5,*}

¹Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan,

²Department of Synthetic Natural Products Chemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan,

³Alzheimer Research Unit, MassGeneral Institute for Neurodegenerative Disease, Massachusetts General Hospital, Charlestown, MA, USA,

⁴Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Tokyo, Japan and ⁵Department of Neuropathology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Amyloid- β peptide ending at the 42nd residue (A β 42) is implicated in the pathogenesis of Alzheimer's disease (AD). Small compounds that exhibit selective lowering effects on A β 42 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 A β 42, possibly by enhancing its conversion to A β 38. 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.

The EMBO Journal (2011) 30, 4815–4824. doi:10.1038/emboj.2011.372; Published online 14 October 2011

Subject Categories: neuroscience; molecular biology of disease

Keywords: Alzheimer; modulation; protease; secretase

Introduction

There is ample evidence supporting the notion that aggregation of amyloid- β peptides (A β) underlies the pathogenesis of Alzheimer's disease (AD) (reviewed by Holtzman *et al*, 2011). A β 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 A β , which in turn impacts the aggregation property of A β : the C-terminally longer A β 42 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 A β 42 without affecting the total γ -secretase activity opened up a way to the safe and effective lowering of the pathogenic A β 42 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 N β , a proteolytic product of Notch corresponding to A β (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 A β 42-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

Received: 29 May 2011; accepted: 21 September 2011; published online: 14 October 2011

Our results suggest that GSM-1 decreases the production of A β 42 possibly by enhancing its conversion to A β 38, by an allosteric mechanism by binding to the TMD1 of PS1.

Results

GSM-1 affects the A β 38- and A β 42-generating activities of γ -secretase

GSM-1 is a phenylpiperidine-type compound that shows a potent A β 42-lowering effect ($IC_{50} = 0.348 \mu M$ in a cell-based assay) accompanied by an increased production of A β 38 (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 A β species that are cognate precursors for secreted form of A β : A β 40 and A β 42 are successively derived from A β 43/46/49 and A β 45/48, respectively (Qi-Takahara *et al*, 2005; Takami *et al*, 2009). An additional cleavage of A β 42 generates A β 38. To further characterize the effect of GSM-1 on the stepwise γ -secretase cleavage leading to A β secretion, we examined the production of the different A β species (i.e., A β 38, A β 40, A β 42, A β 43, A β 45 and A β 46) in an *in-vitro* assay. The levels of total A β 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 A β 42 generation accompanied by an

increase in A β 38, similarly to the results in the cell-based assay (Figure 1B and C). Notably, generation of A β 45, a hypothetical precursor of A β 42 and A β 38, 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/A β 48/49 and A β 45/46, 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 A β 42 and an increase in that of A β 38, 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 A β 38/42-generating γ -secretase activity. (A) Chemical structure of GSM-1 and its pharmacological effect on a cell-based assay. A β levels in conditioned media from HEK293 cells were analysed by ELISAs ($n = 3$, mean $\pm$ 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 A β species were separated by two types of gels (Qi-Takahara *et al*, 2005). (C) Quantitation of *de novo* generated A β in an *in-vitro* assay ($n = 3$, mean $\pm$ s.d., $*P < 0.01$; $**P < 0.05$ at Student's *t*-test). (D) *De novo* A β 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 A β 42-lowering (IC₅₀ = 3.82 μ M in a cell-based assay) and A β 38-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-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

IC₅₀ values against A β 42 generation (IC₅₀ values: GSM-1, 24.09 μ M (Supplementary Figure S3A and B); L-685,458, 0.59 μ M; pep.11, 0.48 μ M; 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 μ M 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 μ M). The labelling was competed by preincubation with GSM-1 (100 μ M). (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 Ni²⁺-affinity column and analysed by immunoblotting.

Figure 2 GSM-1-BpB harbours A β 42-lowering modulator activity. (A) Chemical structure of GSM-1-BpB and its pharmacological effect on a cell-based assay ($n = 3$, mean $\pm$ 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 μ M), GSM-1 (2 μ M) or GSM-1-BpB (2 μ M). (C) Effects on Notch cleavage of DAPT (10 μ M), GSM-1 (0.3 μ M) or GSM-1-BpB (3 μ M) ($n = 3$, mean $\pm$ 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 μ M) and pep.11 (4.8 μ M)). (B–D) Labelling competition experiments with GSM-1 (240.9 μ M) 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 GSIs (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 A β 42-lowering effect. Among the derivatives, NS-1017 (See Supplementary Scheme 2), which harbours an *N*-benzylamide moiety showed a significant A β 42-raising effect associated with decreased A β 38 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 A β 42 production. We also synthesized a phenylpiperidine-type A β 42-raising photoprobe, GSM-1-amide-BpB (See Supplementary Scheme 3), in which the biotin-benzo-

Figure 5 A β 42-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 $\pm$ s.d., * $P<0.01$ at Student's *t*-test). (B) Immunoblot analysis of secreted A β in conditioned media in the presence of DAPT (10 μ M), GSM-1 (2 μ M) or NS-1017 (2 μ M). (C) Labelling competition experiment with GSM-1 (100 μ M) or NS-1017 (200 μ M) for the GSM-1-BpB labelling of PS1 NTF (1 μ M) using mouse brain microsomes. (D) Chemical structure of GSM-1-amide-BpB and its pharmacological effect on a cell-based assay ($n=3$, mean $\pm$ s.d., * $P<0.01$, ** $P<0.05$ at Student's *t*-test). (E) PAL experiment using GSM-1-amide-BpB (1 μ M) 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 1 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 A β 42/A β 40 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 A β 42-lowering 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 1; 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 (PS1 Δ 79) 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 *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 μ M) or GSM-1 (1 μ M) on secreted A β from DKO cells stably expressing APPNL and PS1 mutants ($n=3$, mean $\pm$ 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 μ M). 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 μ M) 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 μ M). 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-PS1₁₋₁₁₀, but not GST or GST-PS1₂₋₆₅ (0.5 μ g 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 A β 42 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 A β 42-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, A β 42-raising 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 A β 42 (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 A β 42 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 A β 40/43 and A β 38/42 from A β 49 and A β 48, respectively (Qi-Takahara *et al*, 2005; Takami *et al*, 2009). Here, we showed that only the A β 38/42-generating activity was modulated by GSM-1 without affecting the levels of AICD as well as of other A β species including A β 45, the latter being the possible cognate precursor for A β 42. 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 A β 42 upon generation of A β 38 (Takami *et al*, 2009). These results lead us to speculate that the A β 42-lowering GSMs selectively alter the proteolytic activity upon the stepwise cleavage to generate A β 38. 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, A β 40 generation was never affected by GSM-1. Notably, alignments of scissile bonds in the two product lines (i.e., A β 49-46-43-40 and A β 48-45-42-38) 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 A β 42 generation. Thus, the selective modulation of A β 38/42 generation suggests that GSM-1 affects both the catalytic pocket and the recognition mechanism of the helical interface during the processive cleavage of A β 48/45/42.

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 *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 A β 42 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 A β 42 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 A β 42 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-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 (University of Florida). Anti-PS1-NTF 121121 (R&D Systems), anti-nicastrin N1660 (Sigma), anti-APP-CTF (Immuno-Biological Laboratories), anti-Aph-1 α O2C2 (Covance), anti-myc 9B11 (Cell Signaling Technology), anti-human A β 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 Notch Δ E (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 PS1₂₋₆₅ and PS1₁₋₁₁₀ 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 *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/Notch Δ E/EGFP/UAS-firefly luciferase was performed as previously described (Isoo *et al*, 2007; Imamura *et al*, 2009). For secreted A β 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 DFP, 0.5 mM PMSF, 1 μ g/ml TLCK, 1 μ g/ml antipain, 1 μ g/ml leupeptin, 10 μ g/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). IC₅₀ values were calculated by plotting data on Kplot 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 methanethio-sulphonate 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>).

Acknowledgements

We are grateful to Drs R Kopan (Washington University in St Louis), G Thinakaran (The University of Chicago), T Golde (University of Florida), B De Strooper (VIB Leuven), H Fuwa (Tohoku University) and Y Li (Sloan-Kettering Cancer Center), T Kitamura (The University of Tokyo), S Funamoto and Y Ihara (Doshisha University) for valuable reagents, Takeda pharmaceutical company for A β ELISA, and our current and previous laboratory members for helpful discussions and technical assistance. This work was supported in part by grants-in-aid for Young Scientists (S) from the Japan Society for the Promotion of Science (JSPS) (TT), Scientific Research on Priority Areas Research on Pathomechanisms of Brain Disorders from the Ministry of Education, Culture, Sports, Science and Technology, Japan (TT and TI), by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (TT and TI), by Targeted Proteins Research Program of the Japan Science and Technology Corporation (JST) (SY, TF, TT and TI), by Core Research for Evolutional Science and Technology of JST (SY, TF, TT and TI), Japan, and by NIH AG15379 (OB). YO and TH are research fellows of JSPS.

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.

References

Beel AJ, Barrett P, Schnier PD, Hitchcock SA, Bagal D, Sanders CR, Jordan JB (2009) Nonspecificity of binding of γ -secretase

modulators to the amyloid precursor protein. *Biochemistry* **48**: 11837–11839

- Das C, Berezovska O, Diehl TS, Genet C, Buldyrev I, Tsai JY, Hyman BT, Wolfe MS (2003) Designed helical peptides inhibit an intramembrane protease. *J Am Chem Soc* **125**: 11794–11795
- De Strooper B, Vassar R, Golde T (2010) The secretases: enzymes with therapeutic potential in Alzheimer disease. *Nat Rev Neurol* **6**: 99–107
- Exstence A (2010) Alzheimer's failure raises questions about disease-modifying strategies. *Nat Rev Drug Discov* **9**: 749–751
- Fuwa H, Takahashi Y, Konno Y, Watanabe N, Miyashita H, Sasaki M, Natsugari H, Kan T, Fukuyama T, Tomita T, Iwatsubo T (2007) Divergent synthesis of multifunctional molecular probes to elucidate the enzyme specificity of dipeptidic γ -secretase inhibitors. *ACS Chem Biol* **2**: 408–418
- Green RC, Schneider LS, Amato DA, Beelen AP, Wilcock G, Swabb EA, Zavitz KH (2009) Effect of tarenflurbil on cognitive decline and activities of daily living in patients with mild Alzheimer disease: a randomized controlled trial. *JAMA* **302**: 2557–2564
- Hayashi I, Urano Y, Fukuda R, Isoo N, Kodama T, Hamakubo T, Tomita T, Iwatsubo T (2004) Selective reconstitution and recovery of functional γ -secretase complex on budded baculovirus particles. *J Biol Chem* **279**: 38040–38046
- Herreman A, Serneels L, Annaert W, Collen D, Schoonjans L, De Strooper B (2000) Total inactivation of γ -secretase activity in presenilin-deficient embryonic stem cells. *Nat Cell Biol* **2**: 461–462
- Hofmann K, Kiso Y (1976) An approach to the targeted attachment of peptides and proteins to solid supports. *Proc Natl Acad Sci USA* **73**: 3516–3518
- Holtzman DM, Morris JC, Goate AM (2011) Alzheimer's disease: the challenge of the second century. *Sci Transl Med* **3**: 77sr71
- Imamura Y, Watanabe N, Umezawa N, Iwatsubo T, Kato N, Tomita T, Higuchi T (2009) Inhibition of γ -secretase activity by helical β -peptide foldamers. *J Am Chem Soc* **131**: 7353–7359
- Isoo N, Sato C, Miyashita H, Shinohara M, Takasugi N, Morohashi Y, Tsuji S, Tomita T, Iwatsubo T (2007) A β 42 overproduction associated with structural changes in the catalytic pore of γ -secretase: common effects of Pen-2 N-terminal elongation and fenofibrate. *J Biol Chem* **282**: 12388–12396
- Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y (1994) Visualization of A β 42(43) and A β 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A β 42(43). *Neuron* **13**: 45–53
- Kakuda N, Funamoto S, Yagishita S, Takami M, Osawa S, Dohmae N, Ihara Y (2006) Equimolar production of amyloid beta-protein and amyloid precursor protein intracellular domain from β -carboxyl-terminal fragment by γ -secretase. *J Biol Chem* **281**: 14776–14786
- Kitamura T, Koshino Y, Shibata F, Oki T, Nakajima H, Nosaka T, Kumagai H (2003) Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. *Exp Hematol* **31**: 1007–1014
- Kopan R, Schroeter EH, Weintraub H, Nye JS (1996) Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain. *Proc Natl Acad Sci USA* **93**: 1683–1688
- Kounnas MZ, Danks AM, Cheng S, Tyree C, Ackerman E, Zhang X, Ahn K, Nguyen P, Comer D, Mao L, Yu C, Pleynt D, Digregorio PJ, Velicelebi G, Stauderman KA, Comer WT, Mobley WC, Li YM, Sisodia SS, Tanzi RE et al (2010) Modulation of γ -secretase reduces β -amyloid deposition in a transgenic mouse model of Alzheimer's disease. *Neuron* **67**: 769–780
- Kukar TL, Ladd TB, Bann MA, Fraering PC, Narlawar R, Maharvi GM, Healy B, Chapman R, Welzel AT, Price RW, Moore B, Rangachari V, Cusack B, Eriksen J, Jansen-West K, Verbeeck C, Yager D, Eckman C, Ye W, Sagi S et al (2008) Substrate-targeting {gamma}-secretase modulators. *Nature* **453**: 925–929
- Li YM, Xu M, Lai MT, Huang Q, Castro JL, DiMuzio-Mower J, Harrison T, Lellis C, Nadin A, Neduvellil JG, Register RB, Sardana MK, Shearman MS, Smith AL, Shi XP, Yin KC, Shafer JA, Gardell SJ (2000) Photoactivated γ -secretase inhibitors directed to the active site covalently label presenilin 1. *Nature* **405**: 689–694
- Lleo A, Berezovska O, Herl L, Raju S, Deng A, Bacskaï BJ, Frosch MP, Irizarry M, Hyman BT (2004) Nonsteroidal anti-inflammatory drugs lower A β 42 and change presenilin 1 conformation. *Nat Med* **10**: 1065–1066
- Morohashi Y, Kan T, Tominari Y, Fuwa H, Okamura Y, Watanabe N, Sato C, Natsugari H, Fukuyama T, Iwatsubo T, Tomita T (2006) C-terminal fragment of presenilin is the molecular target of a dipeptidic γ -secretase-specific inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester). *J Biol Chem* **281**: 14670–14676
- Ogura T, Mio K, Hayashi I, Miyashita H, Fukuda R, Kopan R, Kodama T, Hamakubo T, Iwatsubo T, Tomita T, Sato C (2006) Three-dimensional structure of the γ -secretase complex. *Biochem Biophys Res Commun* **343**: 525–534
- Okochi M, Fukumori A, Jiang J, Itoh N, Kimura R, Steiner H, Haass C, Tagami S, Takeda M (2006) Secretion of the Notch-1 A β -like peptide during Notch signaling. *J Biol Chem* **281**: 7890–7898
- Osawa S, Funamoto S, Nobuhara M, Wada-Kakuda S, Shimojo M, Yagishita S, Ihara Y (2008) Phosphoinositides suppress γ -secretase in both the detergent-soluble and -insoluble states. *J Biol Chem* **283**: 19283–19292
- Page RM, Baumann K, Tomioka M, Perez-Revuelta BI, Fukumori A, Jacobsen H, Flohr A, Luebberts T, Ozmen L, Steiner H, Haass C (2008) Generation of A β 38 and A β 42 is independently and differentially affected by familial Alzheimer disease-associated presenilin mutations and γ -secretase modulation. *J Biol Chem* **283**: 677–683
- Page RM, Gutsmedl A, Fukumori A, Winkler E, Haass C, Steiner H (2010) β -amyloid precursor protein mutants respond to γ -secretase modulators. *J Biol Chem* **285**: 17798–17810
- Qi-Takahara Y, Morishima-Kawashima M, Tanimura Y, Dolios G, Hirokoshi Y, Horikoshi Y, Kametani F, Maeda M, Saido TC, Wang R, Ihara Y (2005) Longer forms of amyloid beta protein: implications for the mechanism of intramembrane cleavage by γ -secretase. *J Neurosci* **25**: 436–445
- Richter L, Munter LM, Ness J, Hildebrand PW, Dasari M, Unterreithmeier S, Bulic B, Beyermann M, Gust R, Reif B, Weggen S, Langosch D, Multhaup G (2010) Amyloid β 42 peptide (A β 42)-lowering compounds directly bind to A β and interfere with amyloid precursor protein (APP) transmembrane dimerization. *Proc Natl Acad Sci USA* **107**: 14597–14602
- Sato C, Morohashi Y, Tomita T, Iwatsubo T (2006) Structure of the catalytic pore of γ -secretase probed by the accessibility of substituted cysteines. *J Neurosci* **26**: 12081–12088
- Sato C, Takagi S, Tomita T, Iwatsubo T (2008) The C-terminal PAL motif and transmembrane domain 9 of presenilin 1 are involved in the formation of the catalytic pore of the γ -secretase. *J Neurosci* **28**: 6264–6271
- Sato T, Nyborg AC, Iwata N, Diehl TS, Saido TC, Golde TE, Wolfe MS (2006) Signal peptide peptidase: biochemical properties and modulation by nonsteroidal antiinflammatory drugs. *Biochemistry* **45**: 8649–8656
- Takagi S, Tominaga A, Sato C, Tomita T, Iwatsubo T (2010) Participation of transmembrane domain 1 of presenilin 1 in the catalytic pore structure of the γ -secretase. *J Neurosci* **30**: 15943–15950
- Takahashi Y, Hayashi I, Tominari Y, Rikimaru K, Morohashi Y, Kan T, Natsugari H, Fukuyama T, Tomita T, Iwatsubo T (2003) Sulindac sulfide is a noncompetitive γ -secretase inhibitor that preferentially reduces A β 42 generation. *J Biol Chem* **278**: 18664–18670
- Takami M, Nagashima Y, Sano Y, Ishihara S, Morishima-Kawashima M, Funamoto S, Ihara Y (2009) γ -Secretase: successive tripeptide and tetrapeptide release from the transmembrane domain of β -carboxyl terminal fragment. *J Neurosci* **29**: 13042–13052
- Takasugi N, Tomita T, Hayashi I, Tsuruoka M, Niimura M, Takahashi Y, Thinakaran G, Iwatsubo T (2003) The role of presenilin cofactors in the γ -secretase complex. *Nature* **422**: 438–441
- Tomita T, Maruyama K, Saido TC, Kume H, Shinozaki K, Tokuhiko S, Capell A, Walter J, Grunberg J, Haass C, Iwatsubo T, Obata K (1997) The presenilin 2 mutation (N141I) linked to familial Alzheimer disease (Volga German families) increases the secretion of amyloid β protein ending at the 42nd (or 43rd) residue. *Proc Natl Acad Sci USA* **94**: 2025–2030
- Tomita T (2009) Secretase inhibitors and modulators for Alzheimer's disease treatment. *Expert Rev Neurother* **9**: 661–679
- Tomita T, Takikawa R, Koyama A, Morohashi Y, Takasugi N, Saido TC, Maruyama K, Iwatsubo T (1999) C terminus of presenilin is required for overproduction of amyloidogenic A β 42 through stabilization and endoproteolysis of presenilin. *J Neurosci* **19**: 10627–10634

- Uemura K, Farner KC, Hashimoto T, Nasser-Ghods N, Wolfe MS, Koo EH, Hyman BT, Berezovska O (2010) Substrate docking to γ -secretase allows access of γ -secretase modulators to an allosteric site. *Nat Commun* **1**: 130
- Uemura K, Lill CM, Li X, Peters JA, Ivanov A, Fan Z, DeStrooper B, Bacskaï BJ, Hyman BT, Berezovska O (2009) Allosteric modulation of PS1/ γ -secretase conformation correlates with amyloid β (42/40) ratio. *PLoS One* **4**: e7893
- Watanabe N, Takagi S, Tominaga A, Tomita T, Iwatsubo T (2010) Functional analysis of the transmembrane domains of presenilin 1: participation of transmembrane domains 2 and 6 in the formation of initial substrate-binding site of gamma-secretase. *J Biol Chem* **285**: 19738–19746
- Watanabe N, Tomita T, Sato C, Kitamura T, Morohashi Y, Iwatsubo T (2005) Pen-2 is incorporated into the γ -secretase complex through binding to transmembrane domain 4 of presenilin 1. *J Biol Chem* **280**: 41967–41975
- Weggen S, Eriksen JL, Das P, Sagi SA, Wang R, Pietrzik CU, Findlay KA, Smith TE, Murphy MP, Bulter T, Kang DE, Marquez-Sterling N, Golde TE, Koo EH (2001) A subset of NSAIDs lower amyloidogenic A β 42 independently of cyclooxygenase activity. *Nature* **414**: 212–216