the finding that mutations in the *APP* gene at chromosome 21 are minor in total FAD pedigree, presenilin (*PS*)*1* and *2* genes were identified as major causative genes for FAD [38–40]. Currently, over 150 point mutations in the *PS1* and *PS2* genes have been linked to FAD [401]. Using cell-based assays and transgenic mice, it was found that FAD-linked mutation in *PS* genes caused selective increase in Aβ42 production [41–45]. By contrast, cells derived from *PS* knockout mice lost γ-secretase activity, suggesting that PS is a pivotal component for γ-secretase activity [46–49]. The development of an *in vitro* γ-secretase assay enabled us to understand that this proteolytic activity was cofractionated with PS polypeptides in high-molecular-weight fractions, suggesting that γ-secretase represents a large protein complex containing PS [50]. Finally, several lines of evidence are emerging to suggest that γ-secretase is a high-molecular-weight, multipass membrane protein complex comprised of PS, nicastrin [51], anterior pharynx-defective 1 (Aph-1) [52,53] and presenilin enhancer 2 (Pen-2) [53] (Figure 2A). Most importantly, ablation of one or the other of these genes resulted in the complete loss of γ-secretase activity [53–61]. By contrast, overexpression of these four components reconstituted γ-secretase activity in heterologous systems [59,62–65], suggesting that these four membrane proteins are the 'core' components of the γ-secretase complex (Figure 2A) [37].

Extensive cellular, molecular and biochemical analyses revealed that PS functions as a catalytic center of γ-secretase, the two intramembranous aspartates within TMD6 and 7 of PS being indispensable for proteolytic activity [66]. Sequence alignments demonstrated that these aspartates are highly conserved among species. Moreover, a γ-secretase inhibitor (GSI) carrying the transition-state analogue moiety directly targeted the PS polypeptides [67,68]. Finally, a discovery of signal peptide peptidase (SPP), which also cleaves membrane-embedded polypeptides with the catalytic aspartates within TMDs [69], enabled classification of these atypical proteases as 'GxGD-type' intramembrane-cleaving aspartic protease [70]. To date, other intramembrane-cleaving proteases with different catalytic sites (i.e., site-2 protease [S2P] as metalloprotease and rhomboid as serine protease) have also been identified [71,72]. These discoveries established the existence of an intramembrane cleaving protease-mediated cellular signaling mechanism termed regulated intramembrane proteolysis [73,74].

In contrast to PS, the functional roles of the other components still remain obscure. Nicastrin may play a role in substrate recognition as an exosite of γ-secretase: its ectodomain can directly interact with the N-terminus of γ-secretase substrates [75], although this view has been challenged [76]. Rather, cell biological analyses indicate that the conformational maturation of the extracellular domain of nicastrin is one of the major determinants for the stabilization and activity of the γ-secretase complex [77]. We recently developed a novel monoclonal antibody targeting the functional conformation of the extracellular domain of nicastrin (patent number WO2007129457 [301]) [78]. Intriguingly, this antibody is able to inhibit γ-secretase activity *in vitro* as well as *in vivo*. Identification of the modes of action of this neutralizing antibody will provide important information about the molecular function of nicastrin. Aph-1 is thought to function as a scaffold together with nicastrin for the γ-secretase complex [79,80]. Pen-2 triggers the activation of the γ-secretase complex and modulates enzymatic activity [59,81]. Moreover, advances in proteomic technology have revealed that the γ-secretase complex contains several other binding partners (e.g., α- and β-catenins [51], CD147 [82] and TMP21 [83]). However, none of these components were copurified with proteolytically active γ-secretase complex [84,85], indicating that the physiological functions of these 'accessory' proteins in intramembrane proteolysis remain to be elucidated. Moreover, the function of other binding partners that were copurified by transition-state analogue GSI still remain unknown [86,87]. Nevertheless, further examinations would provide important information regarding the roles these subunits in γ-secretase activity.

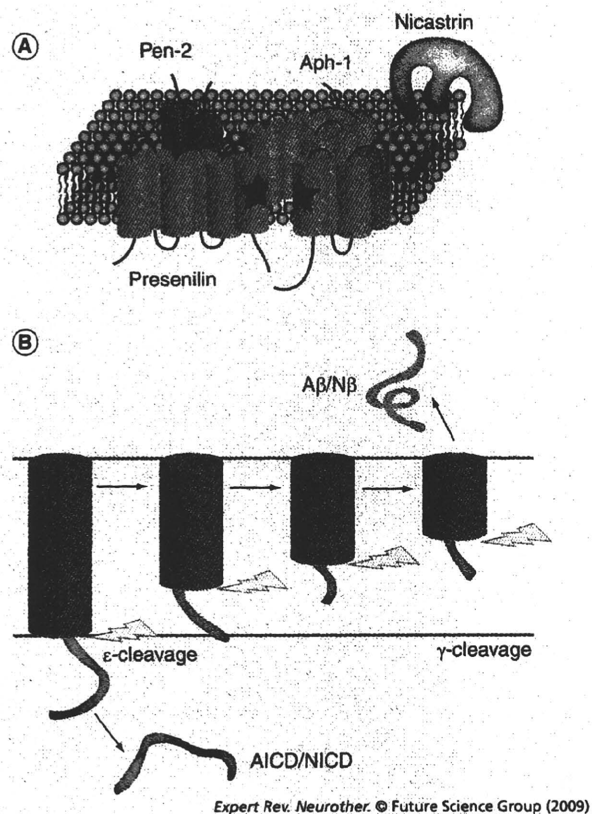

Figure 2. Schematic depiction of the γ-secretase complex and a model of intramembrane cleavage. (A) The core complex of γ-secretase is comprised of Presenilin, Nicastrin, Aph-1 and Pen-2. Presenilin harbors catalytic aspartates (stars) within its transmembrane domains. **(B)** γ-secretase executes intramembrane proteolysis of several single-span membrane proteins, including amyloid β precursor protein and Notch proteins to generate Aβ/AICD and Nβ/NICD, respectively. After ε-cleavage occurred to liberate AICD/NICD, successive cleavage within the transmembrane domain renders to release Aβ/Nβ peptides.
Aβ: Amyloid-β peptide; AICD: Amyloid-β precursor protein intracellular domain; Nβ: Notch-β peptide; NICD: Notch intracellular domain.

Lessons from genetic models for AD therapeutics

The identification of *BACE1* as the protease required for Aβ production in the brain was met with great enthusiasm, particularly when the original analysis of BACE1 knockout mice did not reveal any undesired phenotype with complete loss of brain Aβ [29–31]. However, this enthusiasm has been challenged by thorough analyses of these genetic models. Several investigators identified that *BACE1* knockout mice showed cognitive deficits [88–92], premature death [93] and hypomyelination [94,95]. The hypomyelination phenotype in particuar was caused by a reduction in proteolytic processing of neuregulin-1, the other substrate of BACE1, as well as γ-secretase [96,97]. Moreover, recently dysregulation of neuregulin-1 processing has been shown to cause schizophrenia-like behavioral traits in *BACE1* knockout mice [97,98]. These data suggest that complete inhibition of BACE1 activity might increase the risk for psychological disorder as an adverse effect. However, pharmacological study has suggested that the modulation of BACE1 activity in adults without affecting neuregulin-1 is possible [99]. Moreover, amyloid deposition in the brains of APP transgenic mice carrying a $BACE1^{+/-}$ genotype was significantly decreased [100], indicating that a partial inhibition of BACE1 activity might have a benefit for AD therapeutics.

All FAD-linked mutations in *PS* genes examined so far increased the ratio of Aβ42 that comprises total Aβ. This effect was first observed in human patients [41]. Later, the same effect was observed in *in vitro*, as well as *in vivo*, experimental settings [42–45]. Finally, generation of compound mice expressing FAD-linked *APP* and *PS* genes resulted in an accelerated deposition of amyloid plaques in mice brains [42–44]. By contrast, *PS* knockout in mice brains caused the loss of production of Aβ peptides [46–49]. These data strongly support the amyloid hypothesis: the pathogenic role of Aβ42 and PS/γ-secretase in the deposition of amyloid plaques in brains, thereby causing AD.

Moreover, genetic models provided us with unexpected findings regarding the physiological function of γ-secretase. At first, *PS* mutant *Caenorhabditis elegans* showed significant developmental defects related to Notch signaling [101]. Subsequently, Notch signaling defects were reported in *PS* mutant mice and *Drosophila* [102,103]. The genetic significance of all γ-secretase components in Notch signaling has been found in all genetic models examined so far [53–61,102,103]. Finally, GSI treatment in animals showed a phenocopy of the genetic inhibition of Notch signaling [104–111]. Biochemical studies suggested that Notch, a single-span membrane receptor, is directly cleaved by γ-secretase activity within its TMD (Figure 2B) [112]. The Notch signaling pathway plays a variety of roles in cell fate decisions during development [113,114]. Upon ligand binding from neighboring cells, Notch receptors are proteolytically cleaved by an ADAM/TACE family metalloprotease followed by PS-dependent γ-secretase cleavage to release the Notch intracellular domain (NICD) [115,116]. NICD then translocates into the nucleus and activates transcription of its downstream targets. Up until now, over 50 single-span membrane proteins have been reported as substrates for γ-secretase activity. Some substrates, including Notch, utilize intracellular domains (ICDs) as a transcriptional regulator [117,118]. Notably, the overexpression of FAD-linked mutant human *PS1* rescued the majority of the Notch-related phenotype in *PS1* knockout mice [119], suggesting that the most apparent and conserved 'physiological' function of γ-secretase activity is an essential Notch signaling component. Intriguingly, loss of *PS* genes in mouse brains resulted in the loss of dendritic spines and brain atrophy [120,121]. In addition, some FAD-linked PS mutations decreased the generation of the ICDs and some point mutations on *PS* genes linked to FTD have been found [401]. These data suggest that γ-secretase activity has a seminal role in synaptic maintenance irrespective of Aβ deposition. Importantly, γ-secretase cleaves multiple neuronal substrates to transduce their signals (e.g., ErbB4, p75NTR neurotrophin receptor, N-cadherin and sodium channel β4 subunit) [117,118]. Moreover, recently it was found that FAD-linked mutant *PS1* impairs adult neurogenesis in transgenic mice [122–124]. Nevertheless, the pathological significance of FAD-linked PS mutations on AD through these substrates still remains unknown [125–127].

Development of β-secretase inhibitors

β-secretase is a membrane-bound protease with motifs containing the highly conserved signature sequence of aspartic proteases, D(T/S)G(T/S), within which the aspartic acid residue is essential for proteolytic activity. The crystal structure of BACE1 confirms that the active site is comprised of a long cleft for substrate recognition located between the N- and C-terminal lobes of BACE1 [128]. The side chain pockets located at the surface of the cleft interact with amino acid residues of the substrates, resulting in hydrolysis. Moreover, pH-dependent conformational switching occurs in the active site cleft [129]. These structural features are used for the 'rational design' of β-secretase inhibitors (BSIs) (Figure 3) [17,130]. The first generation of BSIs were designed as transition-state analogues for the aspartyl protease (e.g., OM99-2) (Figure 3 [1]) [128]. In fact, to date, the majority of commercially available potent BSIs are peptide-based compounds containing a transition-state moity (e.g., hydroxyethylene, statin and hydroxymethylcarbonyl isostere). Based on the structural information of BACE1 and transition-state analogue binding, extensive structural modifications aiming at size reduction were performed. Finally, Tang's group at the University of Oklahoma reached a new generation of inhibitors containing hydroxyethylamine isostere and isophthalamide moiety, GRL-8234 (Figure 3 [2]) [131]. This compound potently inhibited BACE1 activity *in vitro* as well as *in vivo*. Kiso's group at Kyoto Pharmaceutical University also generated a small potent BSI containing a 2,6-pyridinedicarboxylic, chelidamic or chelidonic residue at the P_2 position together with hydroxymethylcarbonyl isostere (e.g., KNI-1027) (Figure 3 [3]) [132].

Considering the usage of BSIs as drugs, making less peptidic compounds is mandatory to obtain sufficient oral absorption and penetration of the BBB. In fact, these two issues have hampered progress in development of BSIs for AD therapeutics for a long time. After extensive structure–activity relationship analyses, a hydroxyethylamino isostere-based inhibitor, GSK188909 (Figure 3 [4]), was described as the first orally bioavailable, nonpeptidic BSI that is able to reduce brain Aβ levels in APP transgenic mice [133]. Importantly, a P-glycoprotein inhibitor is required for a

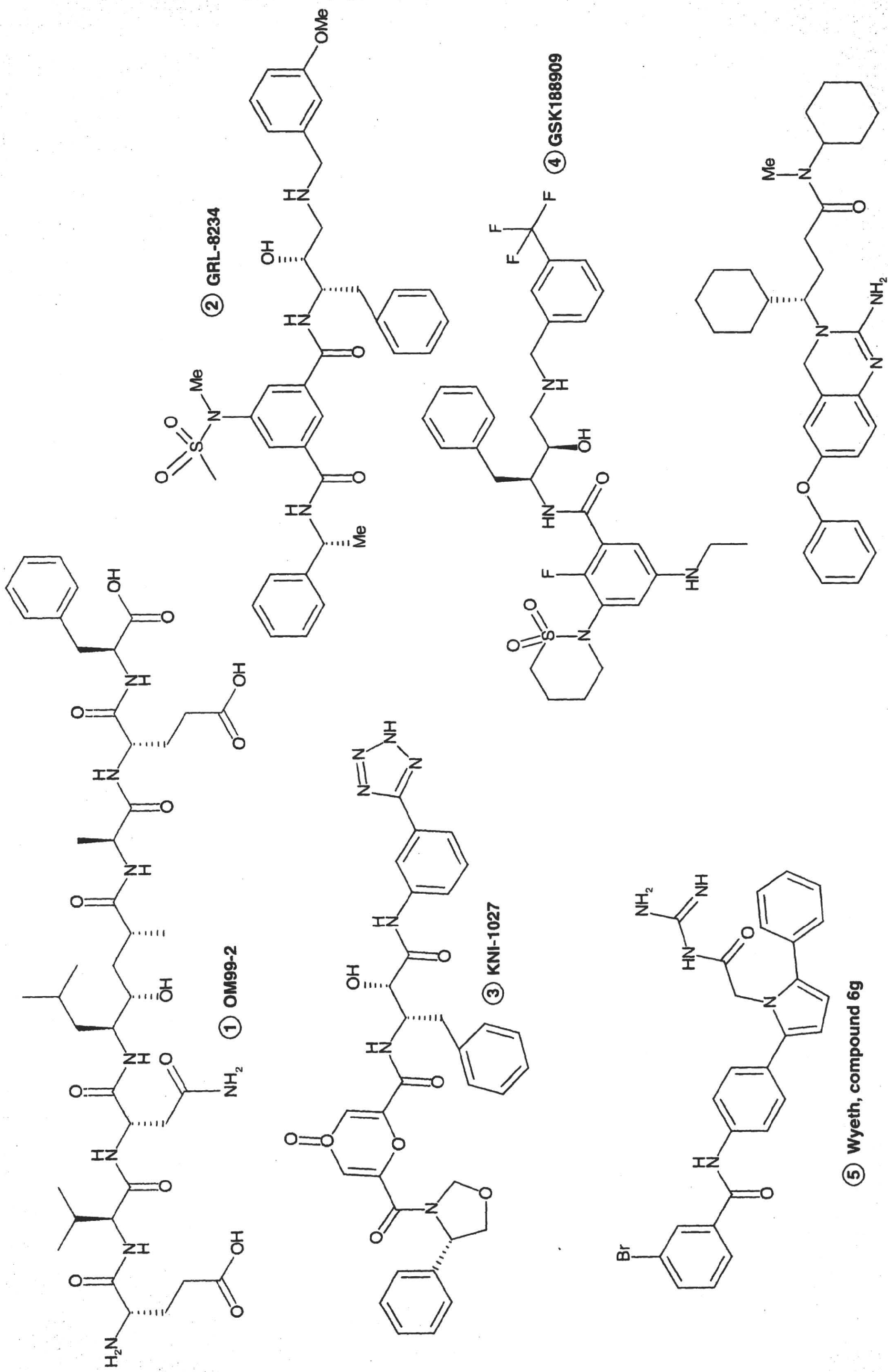

Figure 3. Chemical structures of β-secretase inhibitors.

significant reduction in the levels of Aβ in the brain in an acute oral administration paradigm. However, encouragingly, subchronic dosing of GSK188909 in the absence of a P-glycoprotein inhibitor also lowers brain Aβ. Moreover, high throughput screening using a large compound library by Wyeth led to the discovery of a novel, low-molecular-weight acylguanidine-type BSI (Figure 3 [5]) [134]. The cocrystal structure revealed that the acylguanidine moiety forms hydrogen bonds with the catalytic aspartates of BACE1. Moreover, this inhibitor stabilizes the enzyme in an open conformation, while most peptidomimetic BSIs bind BACE1 in a closed-flap form. Other library screening by Johnson & Johnson also revealed that aminoquinazoline-type compounds (Figure 3 [6]) are able to function as BSIs in a similar manner to that of acylguanidine-type BSIs [135]. Nonetheless, significant advances have been made in the last few years for development of BSI. Most importantly, a drug candidate BSI, CTS-21166, developed by CoMentis and Asteras Pharma has completed a Phase I clinical trial (clinicaltrials.gov identifier NCT00621010).

Development of γ-secretase inhibitors & modulators

Despite the intricacy of γ-secretase and its proteolytic mechanism, several cell-permeable GSIs have been identified by cell-based, high-throughput screening and combinatorial chemistry (Figure 4) [18–21]. Some of the potent peptidic inhibitors include aspartyl protease transition-state mimic moieties (e.g., L-685,458, 31C-III; Figure 4 [7] & [8], respectively), which directly targets the active site of the enzyme [67,68]. They block the cleavage of not only APP but other substrates, such as Notch. However, these transition-state analogues are used only for the discovery stage because of instability and inefficacy in *in vivo*. Importantly, discovery and chemical biological application of these transition-state analogues led to the conclusion that PS is a catalytic subunit in the γ-secretase complex, that is aspartyl protease. Dipeptidic GSIs (e.g., N-[N-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine t-butyl ester [DAPT]; Figure 4 [9]), the potent and cell-permeable compounds, also inhibit Notch signaling. In fact, administration of potent GSIs (e.g., compound E [Figure 4; 10] and LY411575 [Figure 4; 11]) caused significant thymic and gastrointestinal toxicity in mice and rats [104,105,109–111,136]. However, upon extensive investigations by organic chemistry and pharmacological analyses, one of the dipeptidic compounds, called LY450139 (Semagacestat) (Figure 4 [12]) was established by Eli Lilly [137–140]. LY450139 is a potent GSI that shows a good pharmacokinetic profile *in vivo*. LY450139 is now is being tested in a Phase III study (the Interrupting Alzheimer's Dementia by Evaluating Treatment of Amylold Pathology [IDENTITY] trial: clinicaltrials.gov identifier NCT00762411) to see if it can slow the progression of amyloid pathology and neurodegeneration.

The other clue for the modulation of γ-secretase activity by small compounds was recognized from epidemiological studies. A population-based cohort study provided strong evidence that the long-term use of NSAIDs significantly reduced the risk of AD [141]. Disappointingly, a placebo-controlled, randomized trial using naproxen, rofecoxib and cerecoxib, which are US FDA-approved NSAIDs (the Alzheimer's Disease Anti-Inflammatory Prevention Trial [ADAPT]: clinicaltrials.gov identifier NCT00007189), revealed that the anti-inflammatory effect of NSAIDs is not sufficient for the treatment of AD [142,143]. However, molecular and biochemical study revealed that a subset of FDA-approved NSAIDs (e.g., sulindac sulfide (Figure 5 [13]), ibuprofen and indomethacin) directly modulate γ-secretase activity to selectively decrease the secretion of Aβ42 accompanied by an increase in Aβ38 generation, whereas the generation of ICDs was not significantly affected [144–148]. Such compounds are now called γ-secretase modulators (GSMs) (Figure 5) and are expected to be useful as therapeutics for AD without any Notch-based adverse effects. Interestingly, some NSAID- and lipid metabolism-related compounds (e.g., fenofibrate; Figure 5 [14]) caused a significant increase in Aβ42 levels accompanied with a decrease in Aβ38 generation [149,150]. These compounds are called Aβ42-raising GSMs or inverse GSMs. Structure and activity relationship analyses indicate that small modifications to the GSM reverses its character to an Aβ42 raiser, implicating very stringent regulation in the mode of Aβ38 and Aβ42 generation [151,152]. The NSAID-derived Aβ42-selective lowering reagent, *R*-Flurbiprofen (Tarenflurbil) (Figure 5 [15]) [145,146,153] was tested in a Phase III trial (clinicaltrials.gov identifier NCT00322036) by Myriad pharmaceuticals. However, recently this trial was terminated because the drug failed to achieve significance in this study, and the development of this compound was discontinued. However, there are several upcoming potent orally available GSM candidates: CHF5074 (Figure 5 [16]) [154], NGX series compound (Figure 5 [17]) by TorreyPines Therapeutics and GSM-1 (Figure 5 [18]) by Merck [155].

γ-secretase inhibitors with substrate or γ-cleavage site specificity (i.e., second-generation GSIs) might be good candidates for AD drugs that overcome the adverse effects associated with first-generation GSIs *in vivo* (Figure 6). Several pharmaceutical companies published patents regarding compounds containing the sulfonamide moiety as GSIs. The sulfonamide derivative BMS-299897 (Figure 6 [19]) inhibited γ-secretase activity *in vitro* as well as *in vivo* [156], and did not compete with transition-state analogues [157–159], suggesting that sulfonamide-type compounds directly affect the γ-secretase complex at a distinct site from those in which transition-state analogues interact. Notably, BMS-299897 showed no effect on T-cell differentiation, while it reduced Aβ levels in the brains and plasma of transgenic models [110,156], and as a result was classed as a Notch-sparing GSI. Thus, the molecular target/mechanism of Notch-sparing GSIs might make them good candidates for AD treatment. Recently, the other sulfonamide-type Notch-sparing GSI, GSI-953 (Figure 6 [20]) (Begacestat), was developed by Wyeth [160,161], and they announced that they have started Phase I studies (clinicaltrials.gov identifiers NCT00441987, NCT00479219 and NCT00547560).

Alternative approaches for modulation of β- & γ-secretase activities

As BACE1 cleaves APP at the extracellular side, antibody-based novel therapeutic interventions were reported. Administration or overexpression of antibodies against the β-secretase cleavage site of APP caused a significant reduction in Aβ generation [162,163]. These antibodies masked the cleavage site at the ectodomain of APP and caused a steric hindrance of APP–BACE1 recognition.

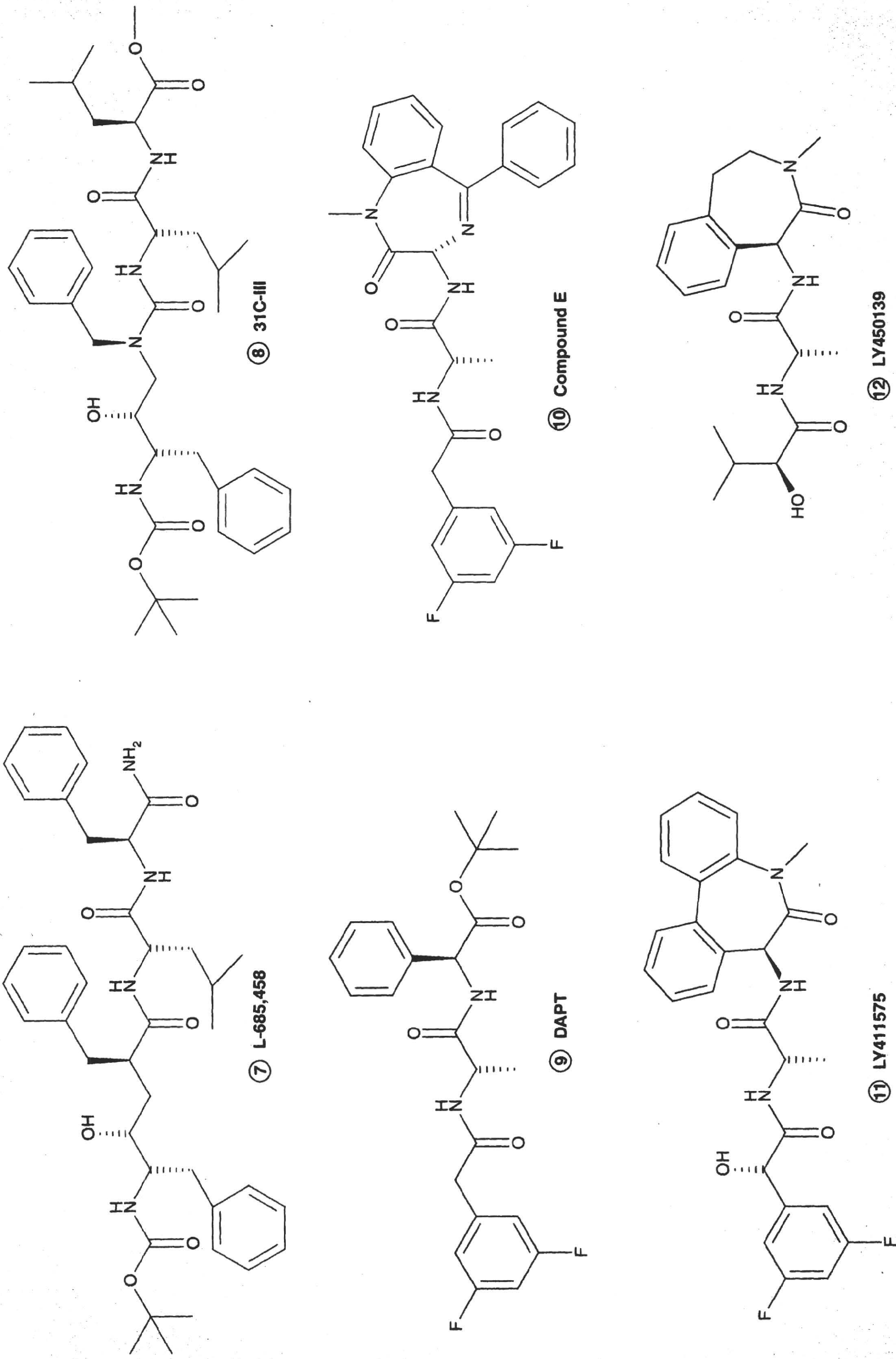

Figure 4. Chemical structures of first-generation γ-secretase inhibitors.

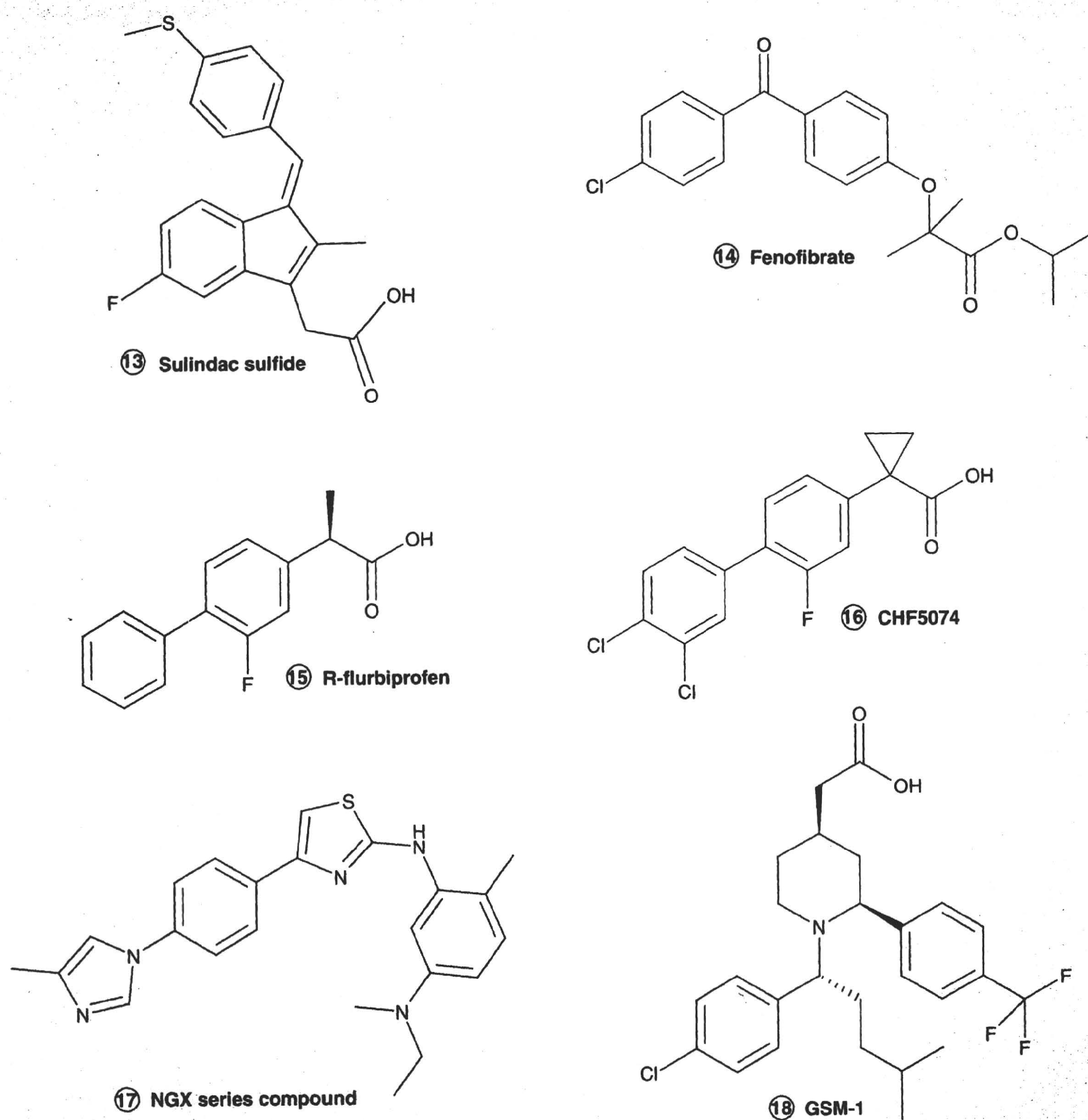

Figure 5. γ-secretase modulators.

Success of this strategy suggests that a compound targeting the β-secretase cleavage site may have a therapeutic benefit for AD. Moreover, Tang's group reported that immunization of BACE1 recombinant protein in an AD mouse model reduced Aβ generation and deposition of amyloid in the mice brains [164]. The neutralizing antibody in this approach specifically bound to BACE1 and serves as a BSI. Identification of the modes of action of this functional antibody would provide novel molecular information regarding the inhibition or modulation of β-secretase activity.

Pathologically, BACE1 protein levels and/or activity have been found to be increased in sporadic AD patients [32–36], while the number of neurons that are responsible for BACE1 expression is low. Thus, direct reduction of BACE1 protein is one of the plausible approaches for therapeutics. The mechanisms by which the expression levels of BACE1 are increased in the AD brain still remain obscure. Importantly, the expression of BACE1 protein is regulated by multiple biological processes in several cell/animal models. In the translation process, energy deprivation induces phosphorylation of the initiation factor eIF2α, which increases translation of BACE1 mRNA and elevates Aβ production [165]. In addition, noncoding RNAs, including BACE1 antisense transcript and several miRNAs, are reported as positive/negative regulators for BACE1 expression via the control of mRNA levels or translation of BACE1 [166–169], suggesting this complicated mechanism

would work in the regulation of BACE1 protein levels. Contrary to BACE1, little contribution of the translational/transcriptional system on the γ-secretase complex is reported. Biochemical studies indicate that γ-secretase is a highly stable protein complex (half-life > 48 h) [170,171]. Moreover, augmentation of γ-secretase activity requires simultaneous overexpression of the four core subunits [59,62–65]. In fact, overexpression of *PS* resulted in the accumulation of PS holoprotein, an inactive form, as the levels of other subunits are limited [172]. Finally, cells derived from compound knockout mice $PS1^{+/-}$ and $PS2^{-/-}$ showed similar levels of γ-secretase activity to that from wild-type mice, suggesting that a single *PS1* gene might be enough for full activity [48,49,173,174]. However, the challenge would shed light on the way to discover a novel molecular target for the effective reduction of Aβ in the brains.

Each of BACE1, γ-secretase and APP extensively undergo intracellular trafficking during APP proteolysis [175,176]. Essentially, acidic pH is the optimal condition for BACE1. Thus, the endocytic process of APP and BACE1 would be required for β-secretase cleavage. This endocytic process of BACE1 required the participation of a family of adaptor Golgi-localizing, γ-adaptin ear homology domain, ARF-binding (GGA) proteins, and reduction of the GGA proteins caused the increase in Aβ production [177]. By contrast, the subcellular localization of functional γ-secretase complex remains unknown. Initial reports using an overexpression paradigm of PS raised a lengthy argument called 'spatial paradox'; overexpressed PS mainly localized at the endoplasmic reticulum, whereas γ-secretase-mediated cleavage would occur distal to this organelle [178]. However, after the identification of nicastrin, which is heavily glycosylated and detected at the cell surface [179], it is now widely accepted that the major functional form of the γ-secretase complex localizes at late secretory and endocytic pathways [180]. Of note, γ-secretase activity showed a distinct cleavage profile at neutral and acidic pH in an *in vitro* assay [181]. In addition, recently, the orphan G protein-coupled receptor (GPR)3 was identified as a genetic modulator of Aβ production [182]. The overexpression of *GPR3* caused an increase in the formation and cell-surface localization of the mature γ-secretase complex without an effect on Notch processing. Thus, subcellular trafficking machinery might modulate the proteolytic activity of the γ-secretase complex.

Moreover, biochemical analyses revealed that APP, BACE1 and γ-secretase were fractionated in lipid raft fractions [183]. The lipid raft is a specialized membrane microdomain that is rich in cholesterol and sphingolipids, and participates in a variety of important biological functions [184,185]. Growing evidence suggests that lipid rafts might be the principal membrane domains in which amyloidogenic processing of APP occurs [175]. Thus, regulating the trafficking mechanisms that enable APP, BACE1 and γ-secretase to localize lipid rafts might provide a novel approach for the reduction of Aβ levels. Recently, it was found that the syntaxin1-X11/Mint-Munc18 axis is involved in the localization of APP in lipid rafts [186]. Moreover, the metabolism of cholesterol, which is an important component of lipid rafts, seems to be involved in the regulation of Aβ generation via direct effects on the amyloidogenic processing. Nevertheless, identification of the components involved in the elusive trafficking mechanisms as well as lipid metabolism responsible for raft localization of APP, BACE1 and γ-secretase could provide a strong impetus for the development of novel therapeutics based on the targeting of APP processing.

As the nonamyloidogenic pathway by α-secretase precludes Aβ formation, an upregulation of α-secretase activity is an alternative strategy to decrease Aβ levels in the brain. In fact, overexpression or stimulation of ADAM10 in mice successfully decreased the Aβ deposition in the brain [187]. Moreover, recently etazolate, a selective $GABA_A$ receptor modulator, was reported as a stimulator for α-secretase activity in neurons [188]. Cell biological study indicates that etazolate showed a neuroprotective effect through increased production of sAPPα. Clinical development of this compound is now underway by ExonHit Therapeutics (Paris, France).

Expert commentary: rational design of GSIs/GSMs

Despite a growing understanding of PS biology, critical questions still remain: how does PS select and transfer the substrates into its active site? How does PS 'hydrolyze' a peptide bond within a hydrophobic environment? How do GSIs/GSMs affect γ-secretase activity to alter Aβ production? All these fundamental questions about intramembrane proteolysis should be answered for rational design of GSIs/GSMs. Biochemical analyses using cell-based and *in vitro* assays provide important

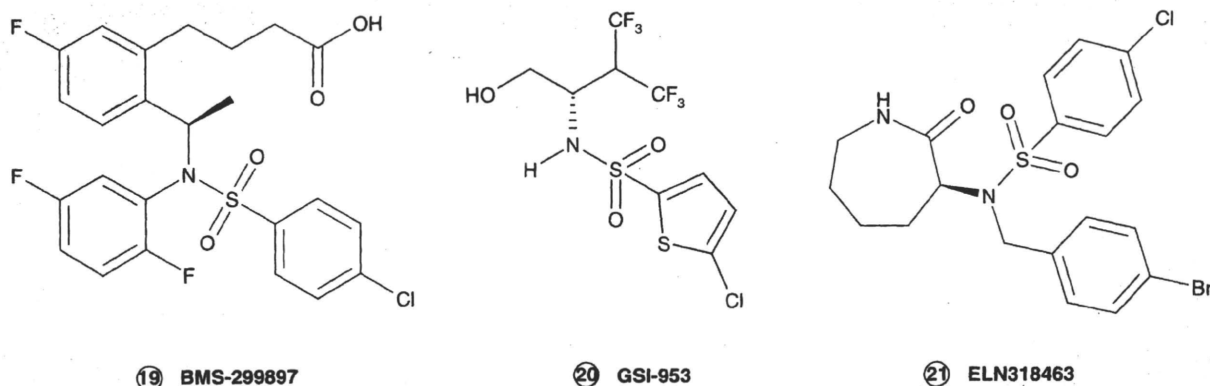

Figure 6. Second-generation γ-secretase inhibitors.

information about the nature of this atypical cleavage. The first finding was that most N-terminals of NICD correspond to an intramembrane site proximal to the cytoplasmic side [115]. Next, APP ICD (AICD) generation from APP was confirmed in a similar manner to that in Notch [189–192]. However, the cleavage site for AICD, called the ε-cleavage site, also locates near the cytoplasmic region where it is distal to the C-terminus of Aβ. Subsequently, γ-secretase cleavage of Notch near the middle of the TMD to release Aβ-like fragments (Notch β) was found, suggesting that both APP and Notch are cleaved at γ- and ε-cleavage sites with different topologies [193]. Genetic and pharmacological analyses indicated that both cleavages are executed by γ-secretase. However, it was very difficult to envision the molecular mechanism of this dual cleavage by a single enzyme, as there is no homology of primary sequence around the γ- and ε-cleavage sites. Key to this unifying idea on dual cleavage is the finding that γ-secretase apparently cuts at the ε-cleavage site first, to produce either a 48- or 49-residue Aβ [194,195]. Subsequent cuts every 3–4 residues (i.e., every helical turn of the substrate) ultimately produce the 38–43 residue Aβ peptides that are secreted from the cell. This mechanism is now conceived as the 'tripeptide hypothesis' of γ-secretase-mediated cleavage (Figure 2B), while the successive model of intramembranous cleavage remains suppositional. Intriguingly, recent biochemical analyses revealed that SPP and SPP-like proteins (SPPLs), that are crossinhibited by a transition-state analogue type GSI, also cleaved at multiple scissile bonds in the substrate [196,197]. This cleavage profile was affected by GSM as well as point mutations in SPP at the conserved residues corresponding to the FAD-linked mutations in PS [196,198,199]. These data indicate that endoproteolysis at several positions in the substrate TMD might be one of the enzymatic features of GxGD-type intramembrane-cleaving aspartic proteases.

Both γ-secretase and SPP showed a broad spectrum for the substrate; no requirement in the primary amino acid sequence was identified [117,118,200–202]. However, existence of helix-breaking residues is required for SPP-mediate cleavage [201]. In addition, helix-dimerization motifs (i.e., GXXXG) significantly affected the property of the γ-secretase cleavage [203–205]. Importantly, the substrate specificities of rhomboid and S2P, the intramembrane-cleaving serine- and metalloproteases, respectively, were also governed by helix-breaking residues [206,207]. These data suggest that the structural conformation of the substrate TMD plays an important role endoproteolysis by intramembrane proteases. However, γ-secretase activity failed to process the substrate for rhomboids in an *in vitro* assay [208]. Moreover, APP processing in cells lacking S2P was unaffected [209]. Finally, sensitivities against protease inhibitors are dependent on the type of catalytic site, suggesting that the cleavage specificity of the intramembrane-cleaving proteases is determined by the structural codes of both substrates and enzymes. Nevertheless, such basic issues require continued attention to acquire a deeper understanding of the various physiological and pathophysiological events mediated by γ-secretase and the other intramembrane-cleaving enzymes.

Identification of the target protein/domain of the GSIs/GSMs by chemical biological strategy would shed light on the molecular function of the subunits in the catalytic process. Photoaffinity probes based on the transition-state analogue type GSI directly targeted PS [67,68], facilitating our understanding that PS itself is a catalytic subunit of the γ-secretase complex. Importantly, the transition-state analogue type GSI copurified the substrate with γ-secretase [86]. Together with enzymological and pharmacological studies [157,158], it is now conceived that PS harbors sites for substrate binding, substrate entry and proteolysis [18,37]. Additionally, a peptide-based approach revealed that TMD1/2 region [210] and the C-terminus of PS, the latter of which is indispensable for γ-secretase complex formation [211–213], have been implicated in the binding of the substrate (patent number WO2004026331) [302]. Understanding the mechanism of substrate recognition and the capture system of γ-secretase is an important issue, as rational development of substrate-specific GSIs is needed for AD therapeutics. It has been demonstrated that the extracellular domain of nicastrin binds to the N-terminus of membrane-bound substrates [75]. However, nicastrin itself has no proteolytic activity. Rather, nicastrin aids substrate recognition for eventual proteolysis by PS as an exosite, although this view has been challenged [76]. Recent genetic analysis using the reconstitution system in yeast also supported an accessory, but not an essential role of nicastrin in γ-secretase-mediated cleavage [214]. Nevertheless, the identification of a γ-secretase neutralizing antibody that targets the extracellular domain of nicastrin suggests that nicastrin might be a novel target for the modulation of γ-secretase activity. Aib-containing helical peptide, which is a TMD mimetic, is able to inhibit γ-secretase activity [215]. Photoaffinity labeling experiments using Aib-containing photoprobe revealed that PS1 N-terminal fragment (NTF) and C-terminal fragment exhibit the helix-binding region, which seems to be a substrate recognition domain in PS [216]. We recently found that a β-peptide-based foldamer that mimics α-helix structure also functions as a potent GSI and targets PS, supporting the notion that γ-secretase recognize the substrate structure (Imamura Y, Watanabe N, Umezawa N *et al.*, Unpublished Data). Further fine protein chemical analysis of the substrate recognition domain might provide useful information for the development of substrate-specific GSIs/GSMs.

We have found that dipeptidic-type GSIs also directly targeted PS [217,218]. Pharmacological experiments suggest that dipeptidic-type GSI inhibits the substrate entry process from the hydrophobic to the hydrophilic environment within the lipid bilayer; the latter corresponds to the catalytic site [108,157–159,216,219]. Intriguingly, even among the dipeptidic-type GSI, DAPT directly targeted the PS C-terminal fragment, but compound E and dibenzazepine bound to PS NTF, while the binding of these compounds completely competed [218]. In addition, pharmacological analyses of these compounds revealed that DAPT is a specific inhibitor for γ-secretase, but compound E and dibenzazepine are able to cross-inhibit SPP and SPPLs [219–221]. No adverse effect caused by the inhibition of the SPP family by

treatment of GSI *in vivo* have thus far been reported. However, proteolytic activities of the SPP family are involved in immune response signals [222], viral and parasite infection [223,224], and inflammatory responses [197,225]. Moreover, SPP mutant animals showed developmental defects [226,227]. Structural analysis of the γ-secretase complex and SPP with these dipeptidic inhibitors would be needed in order to understand the molecular basis of the enzyme specificity.

Currently, much attention is focused on the molecular mechanisms of Notch-sparing GSI. It was reported that sulfonamide-type GSI with different structures (BMS-299897 and ELN318463 [Figure 6; 21]) showed different inhibitory potencies against PS1- and PS2-containing γ-secretase complex, while L-685,458 and DAPT inhibited at similar potencies [228]. Considering the results of competition assay [157–159] and photoaffinity labeling experiments [217,218], sulfonamide-type GSI also targets PS in a similar manner to that by transition state analogues and dipeptidic-type GSI, but the molecular effect(s) might be different. In particular, the PS1-specific property of Notch-sparing GSIs seems to be important for avoiding Notch-based adverse effects. This specificity depends on the primary sequence in TMD3 and the exon 8 region of PS1 [228]. However, the physiological function of this region in the catalytic process still remains unknown. We have developed a sulfonamide-based photoprobe [229] and also found that this compound directly targets PS1 NTF. Investigation of the precise effect of the compound on catalytic processes is now underway.

Finally, recent progress indicates that NSAID-type GSMs as well as an Aβ42-raising GSM, fenofibrate, directly target APP (substrate targeting GSM [stGSM]) [151]. Notably, stGSM interaction was localized to residues 28–36 of Aβ, a region critical for Aβ42 generation as well as the aggregation profile of the peptide [203–205,230]. The compounds known to interact with this region (i.e., Aβ aggregation inhibitors) act as GSMs, and some stGSMs alter the production of cell-derived Aβ oligomers. A similar compound has also been reported by Merck [231]. However, even though the molecular targets of GSMs have been identified, the modes of action of these compounds still remain controversial [81,232].

For mechanism-based rational design of the compounds, understanding the structure–function relationship of the target protein is mandatory. Compared with β-secretase, the γ-secretase complex is one of the most difficult proteins to resolve the structure by conventional structural biological techniques (i.e., x ray crystallography and nuclear MRI), because of the nature of the high-molecular-weight membrane protein complex. However, other approaches are now emerging for the elucidation of the structure and the function of γ-secretase. Substituted cysteine accessibility method (SCAM) is one of the practical approaches to identify the structure of the membrane protein embedded within the lipid bilayer [233,234]. Using SCAM, we and others have identified the existence of a hydrophilic 'catalytic pore' connecting the luminal/extracellular milieu with the catalytic aspartates [235,236]. Moreover, together with the crosslinking and the inhibitor competition assays, we found that TMD9 of PS functions as a lateral gate for the substrate entry from the hydrophobic membranes into the hydrophilic catalytic pore [237,238]. Further examinations by SCAM pave the way for the understanding of the structure of membrane-embedded γ-secretase in an active state.

The other method for understanding the whole structure of the γ-secretase complex is single particle analysis by electron microscopy (EM) of the purified enzyme. Although the resolution of these images is still low, EM analyses have allowed us to recognize an interior pore that is reminiscent of the proteasome and which mediates the entry of water molecules [78,239,240]. Ongoing studies using cryo-EM and/or 2D crystallization may provide fine structural information with higher resolution. In addition, recent progress in structural analyses of intramembrane proteases, rhomboid and S2P protease [241], encourage us to attempt x-ray crystallographic analysis of γ-secretase. Further chemical biological studies, together with structural biology, would be required for the fine understanding of the catalytic mechanisms of γ-secretase and the molecular action of GSIs/GSMs on this atypical enzyme.

Five-year view

Secretases are attractive as molecular targets for AD therapeutics. Significant progress of an effective small molecule-based BSI and GSIs/GSMs have been achieved. Currently, clinical trials using these compounds are ongoing. These proof-of-concept studies using BSIs and GSIs/GSMs lend support to the idea that *in vivo* Aβ reduction can be achieved with this approach, and that the modulation of Aβ levels in the brain is a disease-modifying therapeutic for AD. To make a final conclusion, it may take more than 5 years. However, I strongly believe that we will steadily progress to overcome AD.

Genetic analyses of β- and γ-secretases also provide novel pathological information of other diseases, such as psychiatric disorders and cancer. In particular, the obligatory role of γ-secretase activity in Notch signaling is considered as an obstacle in developing inhibitors for AD therapeutics. However, this problem should be an advantage in chemotherapy of certain cancers, because aberrant Notch signaling has been implicated in several types of cancer [242,243]. Notch signaling also functions in angiogenesis, suggesting that modulation of Notch signaling is applicable to angiogenesis-based cancer therapy [244]. Moreover, Notch signaling is involved in stem cell signaling, including cancer stem cells [245,246]. Notably, it was revealed that combination therapy with a GSI plus glucorticoids can reduce gut toxicity *in vivo* [247]. Thus, the same caveats regarding the significant adverse effects associated with GSIs may be overcome by the development of certain specific compounds and/or effective regimens.

Molecular identification of the secretases has also opened the door for a new basic science: proteolysis of transmembrane proteins around and within the membrane. Common themes have begun to emerge among the evolutionarily diverse class of intramembrane proteases. Complex subcellular traffic and movement between membrane microdomains, following which the formerly separated protease and substrate come into contact, are involved in this

proteolytic event [175,180,248,249]. Proteolytic cleavage is not merely degradative in nature, but initiates a signaling cascade that can impact on genomic expression, cell–cell communication, cellular homeostasis and pathological processes [74]. Nevertheless, further extensive efforts in both academic and pharmaceutical laboratories may raise high hopes for the establishment of a feasible therapeutic approach for AD, psychiatric disease and possibly cancers by the inhibition and modulation of β- and γ-secretase activities.

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Key issues

- The two characteristic pathological feature of Alzheimer's disease (AD) are amyloid plaques and neurofibrillary tangles, the former being composed of amyloid-β peptide (Aβ).
- Multiple rare mutations that cause familial AD lead to increased production or aggregation of Aβ. Aβ is strongly implicated in the pathogenesis of AD.
- Aβ is generated from its precursor protein, amyloid precursor protein, by the sequential action of β- and γ-secretases.
- Inhibition and modulation of β- and γ-secretases to reduce the amount of Aβ in the brain are plausible therapeutics against AD.
- β-site amyloid precursor protein cleaving enzyme, a single membrane-spanning aspartic protease, is responsible for β-secretase activity.
- The γ-secretase complex is an aspartic intramembrane protease comprised of presenilin as a catalytic subunit, nicastrin, anterior pharynx-defective 1 and presenilin enhancer 2.
- Significant progress towards clinical trials has been made in the selective inhibition/modulation of both proteases, regardless of structural information for γ-secretase.
- Several biological aspects and regulatory mechanisms of both secretases have been found, implicating that alternative methods to modulate Aβ-generating activity could be possible.

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Single Chain Variable Fragment against Nicastrin Inhibits the γ-Secretase Activity*[S]

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γ-Secretase is a membrane protein complex that catalyzes intramembrane proteolysis of a variety of substrates including the amyloid β precursor protein of Alzheimer disease. Nicastrin (NCT), a single-pass membrane glycoprotein that harbors a large extracellular domain, is an essential component of the γ-secretase complex. Here we report that overexpression of a single chain variable fragment (scFv) against NCT as an intrabody suppressed the γ-secretase activity. Biochemical analyses revealed that the scFv disrupted the proper folding and the appropriate glycosyl maturation of the endogenous NCT, which are required for the stability of the γ-secretase complex and the intrinsic proteolytic activity, respectively, implicating the dual role of NCT in the γ-secretase complex. Our results also highlight the importance of the calnexin cycle in the functional maturation of the γ-secretase complex. The engineered intrabodies may serve as rationally designed, molecular targeting tools for the discovery of novel actions of the membrane proteins.

γ-Secretase catalyzes intramembrane proteolysis of a variety of substrates including amyloid β precursor protein (APP)[3] to generate amyloid β peptide (Aβ), the latter being a major component of senile plaques in the brains of Alzheimer disease patients. Thus, agents that inhibit γ-secretase activity could serve as an effective therapeutics for Alzheimer disease, whereas the γ-secretase activity plays important roles in cell signaling pathways including Notch signaling (1, 2). γ-Secretase consists of at least four integral membrane proteins, *i.e.* presenilin (PS), nicastrin (NCT), APH-1, and PEN-2, all of which are essential to the proteolytic activity (3–5). Molecular cellular and chemical biological analyses have revealed that PS forms a hydrophilic pore involving the transmembrane domain 6 and 7, where conserved catalytic aspartates reside to function as catalytic residues of γ-secretase complex (6, 7). APH-1 is a multipass membrane protein that plays a role in stabilization and trafficking of the γ-secretase complex (8), and PEN-2 is a cofactor for the activation and the regulation of the γ-secretase activity (3, 9).

NCT, which was identified as a PS-binding protein (10), is a single-pass membrane protein that harbors an extracellular domain (ECD) with a number of *N*-glycosylation sites. In mammalian cells NCT undergoes Endo H-resistant complex glycosylation and acquires trypsin resistance during the assembly process of the γ-secretase complex (11–17). Molecular and cellular analyses revealed that the trypsin resistance, presumably indicating the proper structural folding of NCT, might be directly linked to the enzymatic activity, whereas the complex glycosylation is dispensable. Moreover, multiple sequence alignment analyses revealed that NCT ECD have a similarity to an aminopeptidase (18), whereas certain catalytic residues are not conserved. Recently one study has suggested that NCT plays a critical role in substrate recognition (19). During the proteolytic process, NCT ECD captures the most N terminus of the substrate as a primary substrate receptor (*i.e.* exosite) for the γ-secretase via the aminopeptidase-like domain. However, this view has been recently challenged (20). Nevertheless, as structural information of NCT ECD is totally lacking, the functional role of the structural maturation of NCT in the formation and activity of the γ-secretase remains unclear.

Molecular engineering of monoclonal antibodies opens a venue for the functional analyses of targeted molecule and the therapeutic intervention for several diseases (21). A single-

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[3] The abbreviations used are: APP, amyloid-β precursor protein; Aβ, amyloid-β peptide; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; CNX, calnexin; CST, castanospermine; CTF, C-terminal fragment; NCT, nicastrin; PS, presenilin; Endo H, endoglycosidase H; ECD, extracellular domain; ER, endoplasmic reticulum; scFv, single chain variable fragment.

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chain antibody fragment (scFv) is comprised of heavy- and light-chain sequences of an antibody linked by a short linker and preserves binding abilities of its parental antibody. scFv can be expressed intracellularly as an intrabody (22, 23), which provides a powerful method for phenotypic knock-out of the genes. Intrabodies have been investigated as treatments for a variety of pathological conditions, including neurodegenerative diseases such as Parkinson disease and Huntington disease. Moreover, several recent publications have highlighted the therapeutic potential of intrabodies targeting intra- as well as extracellular epitopes (24–29). Here, we generated scFv against NCT from an anti-NCT monoclonal antibody. Unexpectedly, the overexpression of the anti-NCT scFv as an intrabody abolished the proteolytic activity by the destabilization of the γ-secretase complex and the inappropriate glycosylation of NCT. This is the first example showing that engineered antibody would be a useful tool for the direct modulation of the γ-secretase complex and its activity.

EXPERIMENTAL PROCEDURES

Plasmids—C-terminal V5-His-tagged human NCT ECD inserted in pBlueBac4.5 (Invitrogen) was generated from NCT/V5-His in pBlueBac4.5 (30) by long PCR. Cytoplasmic RNA was prepared from 1×10^7 hybridoma cells by using Isogen reagent (Nippongene, Tokyo, Japan). The cells were lysed by mixing with Isogen and incubated at room temperature for 5 min. After centrifugation of the lysate, the RNA was precipitated and dissolved in distilled water. This RNA was used as a template for first-strand cDNA synthesis with 3′ primers specific for the mouse IgG genes (Novagen, Darmstadt, Germany). The cDNA fragments were then amplified by PCR with LA Taq (Takara, Shiga, Japan) using 3′ and 5′ primers from the mouse Ig primer set as per the manufacturer's instructions (Ig-Prime kit protocols; Novagen). The PCR products were subcloned into the pEF6/V5-His-TOPO vector (Invitrogen) by the TOPO cloning method. scFv cDNAs inserted into pSecTag2C (Invitrogen) were constructed as follows. The PCR-derived DNA fragments in pEF6/V5-His TOPO were subjected to splice overlapping extension PCR to connect heavy- and light-chain genes to give a single fusion protein gene. In the first round PCR, heavy- and light-chain genes were amplified by using the following primers: the variable region heavy-chain gene, 5′-ggggaattcGAAGTGAAGCTGGTGGAG-3′ (VHF#1) and 5′-caccacctccggaaccaccaccaccggaaccaccacctccGGCTGAGGAGACTGTGAGAGT-3′ (VHR#1); the variable region light-chain genes, 5′-ggtggttccggtggtggtggttccggaggtggtggttcaGACATTGTGCTGACACAGTCT-3′ (VLF#1) and 5′-cccgcggccgcTTTTATTTCCAGCTTGGT-3′ (VLR#1) or 5′-ggtggttccggtggtggtggttccggaggtggtggttcaGATATCCAGATGACACAGACT-3′ (VLF#2) and 5′-cccgcggccgcTTTGATTTCCAGCTTGGT-3′ (VLR#2). In the second round PCR, the amplified heavy- and light-chain fragments were linked by using VHF#1 and VLR#1 or #2. The amplified scFv cDNAs were digested with EcoRI and NotI to subclone into the EcoRI-NotI-digested pSecTag2C vector. Wild-type as well as mutant (*i.e.* Δ312, 648ATAA) human NCT inserted in the pEF6/V5-His-TOPO was generated as previously described (14). All cDNAs were sequenced by automated sequencer (LI-COR, Lincoln, NE). cDNAs encoding deletion mutants of human NCT fused with V5 tag were kindly gifted from Drs. Keiro Shirotani and Christian Haass (Ludwig-Maximilians-University, Munich, Germany) (17).

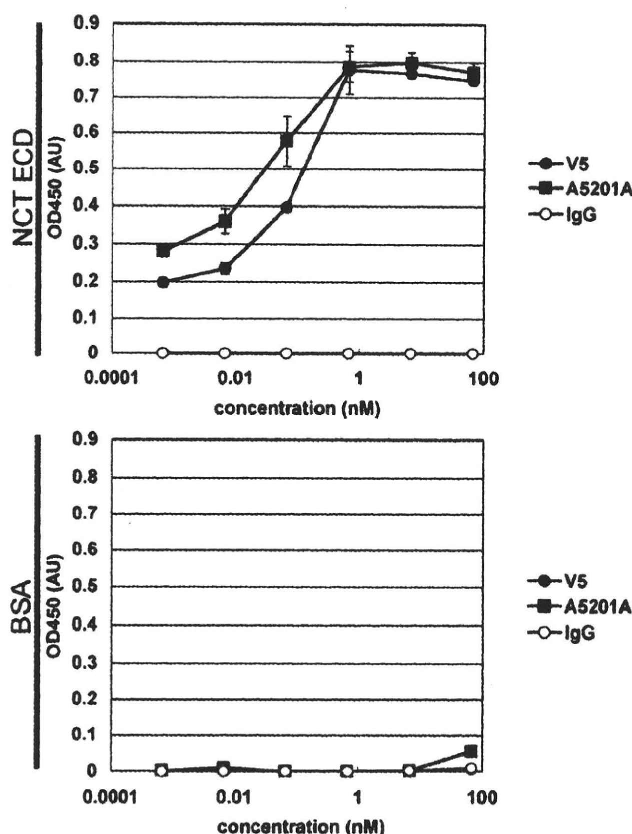

FIGURE 1. **Binding of A5201A to recombinant NCT ECD.** Sequentially diluted A5201A, anti-V5 monoclonal antibody, or mouse IgG fraction was applied to NCT ECD- or bovine serum albumin-coated plates. Subsequently, anti-mouse IgG antibody conjugated with horseradish peroxidase was incubated, and monoclonal antibody binding was quantitated by measuring A_{450} using peroxidase substrate. *AU*, absorbance units.

Cell Culture and Transfection—Maintenance of Sf9 cells, transfection, and recombinant baculovirus preparation were done as previously described (30, 31). Hybridoma cells were maintained in RPMI 1640 medium supplemented with L-glutamine (Nikken Bio Medical Laboratory, Kyoto, Japan) containing 15% (v/v) fetal bovine serum, 100 international units/ml of penicillin, 100 μg/ml of streptomycin, and 1 mM sodium pyruvate (Sigma) at 37 °C in 5% CO_2. All transfections were achieved by FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. HEK293 cell lines stably expressing scFv were selected by Zeocin (Invitrogen). *Ncstn* knock-out fibroblasts (NKO cells) (32) stably expressing wild-type and mutant NCT were selected by Blasticidin (Calbiochem).

Purification of NCT ECD and Secreted scFv—For NCT ECD production, Sf9 cells were infected with recombinant virus encoding NCT ECD at multiplicity of infection 2 and incubated for 72 h. For scFv, 5201F-expressing cells (clone 2) were incubated in regular media for 72 h. NCT ECD or scFv was recovered from the culture media by using a nickel-chelating column (GE Healthcare). Bound proteins were eluted by a stepwise gradient of imidazole (5–300 mM) in phosphate-buffered saline.

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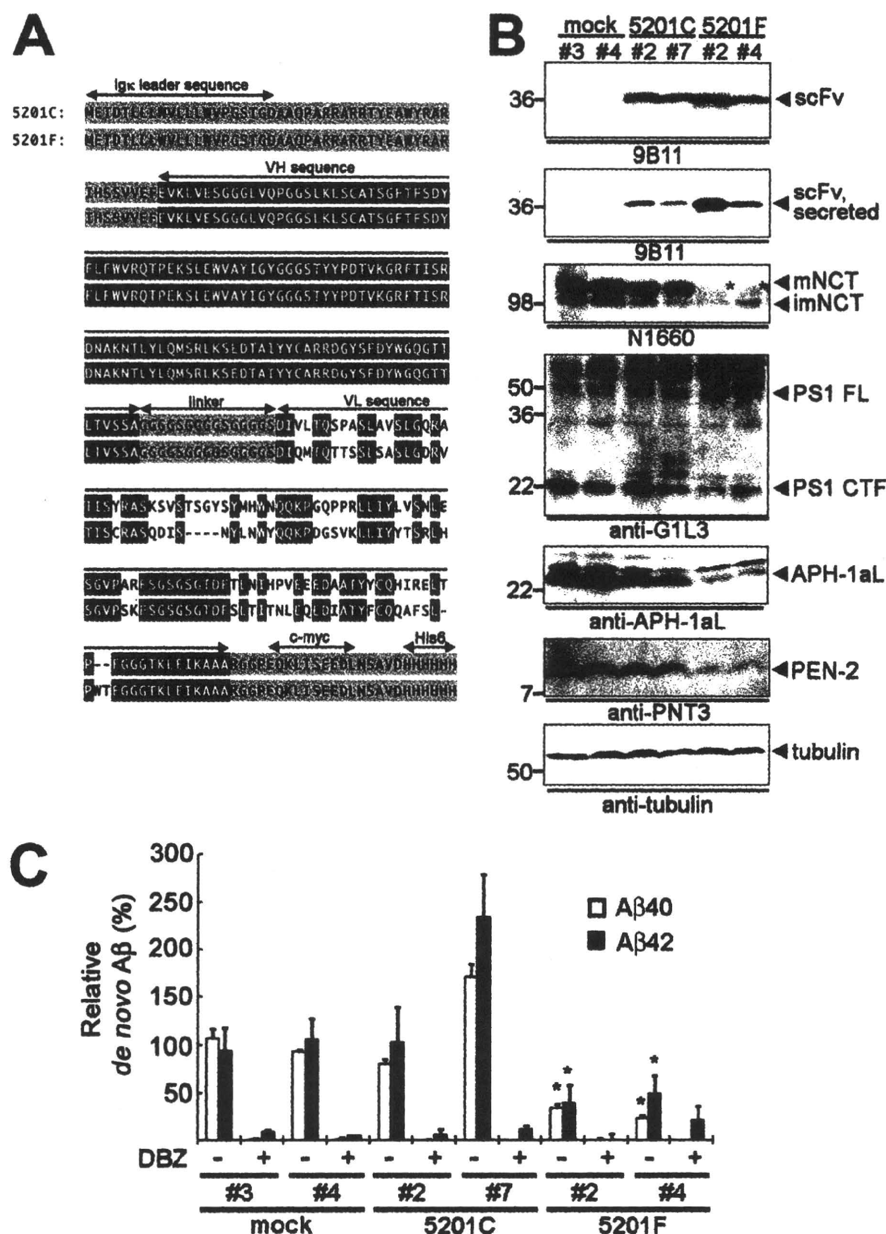

FIGURE 2. **Effects of overexpression of 5201F on the expression levels of the γ-secretase components and proteolytic activity.** *A*, the sequence alignment of the obtained scFvs, 5201C, and 5201F. *B*, immunoblot analysis of HEK293 cells stably expressing intrabodies with each antibody, indicated *below the panel*. *mNCT* and *imNCT* represent mature and immature NCT, respectively. *FL*, full-length. Mature NCT with faster migration in 5201F-expressing cells (NCT*) was indicated by an *asterisk*. *C*, specific γ-secretase activity of the intrabody-expressing cells measured by *in vitro* assay. Solubilized cell membranes were coincubated with the substrates in the presence (+) or absence (−) of 100 nM DBZ. *De novo* generation levels of Aβ40 (*open columns*) or Aβ42 (*filled columns*) peptides were normalized by the γ-secretase levels, which were assessed by densitometric analysis of PS1 CTF on the immunoblotting ($n = 3$, *, $p < 0.01$, Student's *t* test).

Eluted fractions were analyzed by Coomassie or silver staining as well as immunoblotting.

Analysis of A5201A Binding Ability by Enzyme-linked Immunosorbent Assay—Purified NCT ECD or bovine serum albumin was coated on 96-well plates at the concentration of 2 μg/ml in a coating buffer (0.1 M sodium bicarbonate, pH 8.6), and the plates were incubated overnight at 4 °C. After the coating, the plates were blocked by a blocking buffer (phosphate-buffered saline containing 1× BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) and 0.02% (w/v) sodium azide) and stored at 4 °C until used. A5201A, anti-V5 antibody (Invitrogen), as a positive control or mouse IgG fraction (SIGMA) as a negative control was added at various concentrations to the wells, and the plates were incubated overnight at 4 °C. Binding of antibody was detected by anti-mouse IgG antibody conjugated with horseradish peroxidase (GE Healthcare) and tobacco mosaic virus substrate. A_{450} was measured and quantitated by SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA).

Antibodies, Immunological Analyses, and in Vitro γ-Secretase Assay—Anti-G1Nr3, G1L3, and PNT3 polyclonal antibodies against glutathione *S*-transferase-fused human PS1 N terminus, cytoplasmic loop region, or synthetic peptide corresponding to the N-terminal 26 amino acids of human/mouse PEN-2, respectively, were previously described (30, 33–35). Anti-PS1NT polyclonal antibody was kindly gifted from Drs. Gopal Thinakaran and Sangram Sisodia (The University of Chicago, Chicago, IL). Other antibodies were purchased from Cell Signaling Technology (Danvers, MA) (anti- c-*myc* (9B11)), Covance (Princeton, NJ) (anti-APH-1aL (O2C2)), Santa Cruz Biotechnology (Santa Cruz, CA) (anti-NCT N terminus (N19)), Sigma (anti-NCT C terminus (N1660), anti-α-tubulin (DM1A)), or Stressgen (Ann Arbor, MI) (anti-Calnexin). Cells were solubilized with HEPES buffer (10 mM HEPES, pH 7.4, 150 mM NaCl) containing 1% (w/v) CHAPSO. Immunoprecipitation, immunoblot analysis, metabolic labeling, and enzymatic digestion experiments were previously described (14, 33–36). For detection of the γ-secretase activity *in vitro*, solubilized HeLa cell membranes were co-incubated with the APP-based recombinant substrates in the presence (+) or absence (−) of 100 nM DBZ (YO01027), which was kindly provided from Dr. Haruhiko Fuwa (Tohoku University, Miyagi, Japan) (3, 37–39). Aβ was quantified by human β

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