Emerging structures and dynamic mechanisms of γ-secretase for Alzheimer's disease

Yinglong Miao[1, *], Michael S. Wolfe[2, *]

https://doi.org/10.4103/NRR.NRR-D-23-01781

Date of submission: October 30, 2023

Date of decision: December 19, 2023

Date of acceptance: January 11, 2024

Date of web publication: March 1, 2024

From the Contents

Abstract

γ-Secretase, called "the proteasome of the membrane", is a membrane-embedded protease complex that cleaves 150+ peptide substrates with central roles in biology and medicine, including amyloid precursor protein and the Notch family of cell-surface receptors. Mutations in γ-secretase and amyloid precursor protein lead to early-onset familial Alzheimer's disease. γ-Secretase has thus served as a critical drug target for treating familial Alzheimer's disease and the more common late-onset Alzheimer's disease as well. However, critical gaps remain in understanding the mechanisms of processive proteolysis of substrates, the effects of familial Alzheimer's disease mutations, and allosteric modulation of substrate cleavage by γ-secretase. In this review, we focus on recent studies of structural dynamic mechanisms of γ-secretase. Different mechanisms, including the "Fit-Stay-Trim", "Sliding-Unwinding" and "Tilting-Unwinding", have been proposed for substrate proteolysis of amyloid precursor protein by γ-secretase based on all-atom molecular dynamics simulations. While an incorrect registry of the Notch1 substrate was identified in the cryo-electron microscopy structure of Notch1-bound γ-secretase, molecular dynamics simulations on a resolved model of Notch1-bound γ-secretase that was reconstructed using the amyloid precursor protein-bound γ-secretase as a template successfully captured γ-secretase activation for proper cleavages of both wildtype and mutant Notch, being consistent with biochemical experimental findings. The approach could be potentially applied to decipher the processing mechanisms of various substrates by γ-secretase. In addition, controversy over the effects of familial Alzheimer's disease mutations, particularly the issue of whether they stabilize or destabilize γ-secretase-substrate complexes, is discussed. Finally, an outlook is provided for future studies of γ-secretase, including pathways of substrate binding and product release, effects of modulators on familial Alzheimer's disease mutations of the γ-secretase-substrate complexes. Comprehensive understanding of the functional mechanisms of γ-secretase will greatly facilitate the rational design of effective drug molecules for treating familial Alzheimer's disease and perhaps Alzheimer's disease in general.

Key Words: Alzheimer's disease; amyloid precursor protein; cryo-EM structures; drug design; intramembrane proteolysis; molecular dynamics; Notch

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease, for which the formation of 42-residue amyloid β-peptide ($Aβ_{42}$) plaques in the brain is a defining pathological feature (Selkoe and Hardy, 2016). Aβ peptides are generated through proteolysis of the amyloid precursor protein (APP) by the β-secretase and γ-secretase (GSEC) (Haass et al., 2012). GSEC is considered the "proteasome of the membrane" with over 150 known substrates, including APP and Notch. Proteolysis of APP by GSEC is complex (Steiner et al., 2018). Initial endoproteolysis of the 99-residue C-terminal fragment (C99) of APP (the ε cleavage) by GSEC generates $Aβ_{49}$/$Aβ_{48}$ and the corresponding APP intracellular domains (Gu et al., 2001). These Aβ peptides are then trimmed every 3–4 amino acids by GSEC along two pathways: $Aβ_{48}$→$Aβ_{45}$→$Aβ_{42}$→$Aβ_{38}$ and $Aβ_{49}$→$Aβ_{46}$→$Aβ_{43}$→$Aβ_{40}$ (Qi-Takahara et al., 2005; Takami et al., 2009). Mutations in APP and GSEC associated with early-onset familial AD (FAD) can skew substrate processing to generate the pathological $Aβ_{42}$ (Sato et al., 2003; Bolduc et al., 2016). Critical gaps remain in understanding the mechanisms of intramembrane proteolysis of different substrates by GSEC and the effects of FAD mutations.

GSEC has long been targeted for the potential treatment of AD. However, GSEC inhibitors (GSIs) failed in clinical trials (Doody et al., 2013a), partly due to a lack of selectivity for APP over other substrates such as Notch (Doody et al., 2013a,

[1]Computational Medicine Program and Department of Pharmacology, University of North Carolina – Chapel Hill, Chapel Hill, NC, USA; [2]Department of Medicinal Chemistry, University of Kansas, Lawrence, KS, USA

*__Correspondence to:__ Yinglong Miao, PhD, Yinglong_Miao@med.unc.edu; Michael S. Wolfe, PhD, mswolfe@ku.edu.

https://orcid.org/0000-0003-3714-1395 (Yinglong Miao); https://orcid.org/0000-0002-5721-9092 (Michael S. Wolfe)

Funding: *This work was supported in part by Award 2121063 from National Science Foundation (to YM) and AG66986 from the National Institutes of Health (to MSW).*

How to cite this article: *Miao Y, Wolfe MS (2024) Emerging structures and dynamic mechanisms of γ-secretase for Alzheimer's disease. Neural Regen Res 19(0):000-000.*

b; Coric et al., 2015). However, GSEC modulators (GSMs) offer promising selective treatment for AD (Bursavich et al., 2016). GSMs that selectively stimulate γ-secretase-mediated trimming of $A\beta_{42}$ to $A\beta_{38}$ were first identified in 2001 (Weggen et al., 2001). Since then, hundreds of GSMs have been developed (Wolfe, 2007; Kukar et al., 2008; Bursavich et al., 2016), including early nonsteroidal anti-inflammatory drugs, and second-generation compounds covering a wide range of chemical diversity. Recent GSMs have achieved significantly improved drug-like properties and excellent potency and efficacy, with nanomolar EC50 for reducing $A\beta_{42}$. Nevertheless, very few GSMs such as Tarenflurbil have advanced into clinical trials (Wilcock et al., 2008; Green et al., 2009; Luo and Li, 2022).

Remarkable advances in cryo-electron microscopy (cryo-EM) have led to high-resolution structures of GSEC (Lu et al., 2014; Bai et al., 2015b), which is comprised of four protein components: catalytic subunit presenilin (PS1), nicastrin (NCT), anterior pharynx-defective 1 and presenilin enhancer 2 (**Figure 1A**). The first cryo-EM structure was determined for human GSEC in the substrate-free (apo) form at 3.4 Å resolution in 2015 (PDB: 5A63; Bai et al., 2015b). The structure showed a horseshoe shape of the complex transmembrane (TM) bundle and high flexibility of the PS1 catalytic subunit, with a low-resolution density map of the TM2 and TM6 helices. The conformational plasticity of PS1 was further characterized with three distinct conformations identified for the apo enzyme in a follow-up study (Bai et al., 2015a). Another cryo-EM structure was also reported for GSEC in complex with the N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) dipeptidic inhibitor, which stabilized the TM2 and TM6a helices with their atomic coordinates resolved (PDB: 5FN2; **Figure 1A**). In 2019, two structures were reported for GSEC bound by the APP and Notch substrates, which adopt closely similar conformations (Yang et al., 2019; Zhou et al., 2019). In 2021, a new structure was published for GSEC bound by both the L458 GSI and E2012 GSM (PDB: 7D8X, **Figure 1A**; Yang et al., 2021). The L458 GSI occupies the same location on PS1 that accommodates the β-strand from APP/Notch and directly coordinates with the catalytic aspartate residues. E2012 binds to an allosteric site at the interface between hydrophilic loop 1 of PS1 and NCT, partially capping the extracellular space of the substrate-binding tunnel. The new cryo-EM structures provide extremely valuable insights into the nature of substrate recognition and binding of the GSI and GSM. However, the dynamic mechanism of allosteric modulation of APP cleavage by GSEC remains unclear, hindering the rational design of more effective GSMs for treating AD.

Molecular dynamics (MD) is useful to simulate the structural dynamics of biomolecular complexes (Karplus and McCammon, 2002), such as GSEC. In 2015, the first atomistic MD simulation was performed on isolated PS1 using a homology model, showing that the TM2, TM6, and TM9 were highly mobile (Kong et al., 2015). Since then, computer simulations have provided important insights into the structural dynamics and functional mechanisms of GSEC, including the conformational changes (Somavarapu and Kepp, 2016; Aguayo-Ortiz et al., 2017; Somavarapu and Kepp, 2017; Dehury et al., 2019a; Hitzenberger and Zacharias, 2019b, c), enzyme allosteric modulation (Lee et al., 2017), substrate binding and cleavage (Somavarapu and Kepp, 2017; Dehury et al., 2019a; Götz et al., 2019a, b; Hitzenberger and Zacharias, 2019c; Bhattarai et al., 2020, 2022; Dehury et al., 2020a, b; Chen and Zacharias, 2022; Koch et al., 2023), water distribution (Hitzenberger and Zacharias, 2019b, c; Do et al., 2023a), lipid interactions (Hitzenberger and Zacharias, 2019b), ligand binding of GSIs (Gertsik et al., 2017; Hitzenberger and Zacharias, 2019a; Petit et al., 2019) and GSMs (Mehra and Kepp, 2021) and effects of mutations (Götz et al., 2019a, b; Chen and Zacharias, 2020; Soto-Ospina et al., 2021; Devkota et al., 2024; Do et al., 2023b). An excellent review was published that covered computational studies of the structural dynamics and substrates of GSEC until 2020 (Hitzenberger et al., 2020; **Table 1**). In this review, we focused on more recent studies afterwards about the structures and dynamics of GSEC (**Figure 1B** and **Table 1**).

Table 1 | **Summary of computational dynamics studies of GSEC up to date (October 2023)**

Year	Topics	References
2015	PS1 substrate entry	Kong et al., 2015
2016	PS1 gate dynamics	Somavarapu and Kepp, 2016
2017	GSEC-inhibitor binding	Gertsik et al., 2017
	Structural ensemble of GSEC	Aguayo-Ortiz et al., 2017
	Allosteric modulation of GSEC	Lee et al., 2017
	Different conformations of GSEC	Somavarapu and Kepp, 2017
2019	Loose & compact binding of C99	Dehury et al., 2019a
	Comparison of PS1 and PS2	Dehury et al., 2019b
	APP hinge flexibility, I45T APP mutant	Götz et al., 2019a, b
	Dynamics, Aβn & inhibitor binding of GSEC	Hitzenberger and Zacharias, 2019a, b, c
	APP-NCT interface	Petit et al., 2019
2020	Review: Dynamics and substrates of GSEC	Hitzenberger et al., 2020
2020	GSEC activation and APP cleavage	Bhattarai et al., 2020
	Effects of GSEC mutations	Chen and Zacharias, 2020
	Notch *vs.* APP binding	Dehury et al., 2020a, b
2021	GSEC with APH-1B	Dehury and Kepp, 2021
	GSEC modulators	Mehra and Kepp, 2021
	PS1 mutations	Soto-Ospina et al., 2021
2022	Tripeptide trimming	Bhattarai et al., 2022
	Internal docking site	Chen and Zacharias, 2022
2023	Notch proteolysis	Do et al., 2023b
	APP ectodomain interactions	Koch et al., 2023
	Effects of PS1 FAD mutations	Devkota et al., 2024; Do et al., 2023a

An excellent review was published that covered computational studies of the structural dynamics and substrates of GSEC until 2020 (Hitzenberger et al., 2020). In this review, we focus on more recent studies afterwards about the structures and dynamics of GSEC.

Search Strategy

The PubMed library with EndNote was used for the literature search using the following keywords: gamma-secretase, structures, dynamics and simulations. All years were chosen in the search. These searches were performed between May and October 2023.

Mechanistic Models of Substrate Cleavage by γ-Secretase

Prior to the publication of the cryo-EM structures of substrate-bound GSEC, computer simulations were historically

performed on the substrate-free (apo) GSEC, for which a putative active conformation was characterized by close interaction between the two catalytic aspartates (Aguayo-Ortiz et al., 2017; Hitzenberger and Zacharias, 2019b, c). In addition, the two catalytic aspartates apparently underwent electrostatic repulsion when both were deprotonated with negative charges in the active site (Kong et al., 2015; Dehury et al., 2020a, b; Hitzenberger et al., 2020). Proper proteolysis of the substrates would require one of the catalytic aspartates to be protonated and act as an acid, as well as a catalytic water molecule (Northrop, 2001; Wolfe, 2009).

Early MD simulations of membrane-embedded apo GSEC identified "Closed", "Semi-open" and "Fully-open" conformations of PS1 with respect to the size of the catalytic pocket, which was defined by the distance from catalytic aspartates to the intracellular ends of TM2 and TM3 (**Figure 1C**; Somavarapu and Kepp, 2017). Peptides were then docked into the different conformations of PS1. It was suggested that the substrate must fit into the protein pocket to stay long enough to be trimmed to shorter peptides. Therefore, a "Fit-Stay-Trim" mechanism was proposed by the Kepp group for substrate processing by GSEC (**Figure 1C**; Somavarapu and Kepp, 2017; Mehra and Kepp, 2022).

Based on known experimental structures of apo GSEC and C99, the Zacharias group generated computational models of GSEC-C99 binding in three different possible modes. Restraint MD simulations were then applied to pull the substrate scissile bond into a cleavage transition-like state. The most likely binding mode was predicted for APP with the substrate helix located in a cleft between the TM2 and TM3 helices of PS1, which agreed with the cryo-EM structure of the APP-bound GSEC (PDB: 6IYC; **Figure 1A**). Further restraint MD simulations were performed to produce complex structures of the $A\beta_{49}$-, $A\beta_{46}$- and $A\beta_{43}$-bound GSEC without the Nicastrin subunit. The N-terminus of each Aβ peptide was anchored to maintain its interaction with PS1. Simulations on these complexes showed that both helix unwinding and sliding of active site aspartates towards the scissile amide bond are responsible for peptide repositioning during substrate processing by GSEC. The restraint MD simulations thus suggested a "Sliding-Unwinding" mechanism for substrate processing of APP by GSEC (**Figure 1D**; Hitzenberger and Zacharias, 2019c).

In 2020, we combined Gaussian accelerated Molecular Dynamics (GaMD) simulations and biochemical experiments to investigate substrate processing of wildtype and mutant APP by GSEC (Bhattarai et al., 2020). The cryo-EM structure of APP-bound GSEC was computationally activated (catalytic Asps restored) and artificial Cys mutagenesis and disulfide crosslinking were undone. GaMD simulations captured spontaneous activation of GSEC, with H-bonded catalytic aspartates and water poised for the ε cleavage of APP (**Figure 1E**). Moreover, GaMD simulations revealed that APP FAD mutations I45F and T48P preferred ε cleavage between residues Leu49-Val50, while the M51F mutation shifted ε cleavage site to Thr48-Leu49 (Bhattarai et al., 2020). In a follow-up study (Bhattarai et al., 2022), mass spectrometry and western blotting were used to quantify the efficiency of tripeptide trimming of wildtype and FAD mutant $A\beta_{49}$ to $A\beta_{46}$ (the ζ cleavage). In comparison to wildtype $A\beta_{49}$, the efficiency of tripeptide trimming was similar for the I45F, A42T, and V46F $A\beta_{49}$ FAD mutants, but substantially diminished for the I45T and T48P mutants. In parallel with these biochemical experiments, Peptide GaMD (Pep-GaMD) simulations were applied to investigate tripeptide trimming of $A\beta_{49}$ by GSEC. The starting structure was active GSEC bound to $A\beta_{49}$ and APP intracellular domains, as generated from the previous study (Bhattarai et al., 2020). Pep-GaMD simulations captured remarkable structural rearrangements of both the enzyme and substrate, in which hydrogen-bonded catalytic aspartates and water became poised for tripeptide trimming of $A\beta_{49}$ to $A\beta_{46}$. These structural changes required a positively charged N-terminus of endoproteolytic coproduct APP intracellular domains, which could dissociate during conformational rearrangements of the protease and $A\beta_{49}$. During trimming, two residues unwound from the C-terminus of the $A\beta_{49}$ helical domain, and the latter tilted by ~50 degrees. The complementary GaMD simulations and biochemical experiments suggested a "Tilting-Unwinding" mechanism of substrate processing of APP by GSEC (**Figure 1E**; Bhattarai et al., 2022). As with ε proteolysis, GaMD simulations were in good agreement with biochemical results: I45F, A42T and V46F $A\beta_{49}$ FAD mutants were activated for ζ similar to WT Ab49, while I45T and T48P $A\beta_{49}$ FAD mutants were deficient in this trimming step.

Comparison of Notch and Amyloid Precursor Protein Processing by γ-Secretase

Compared with APP proteolysis, significantly fewer studies have been carried out on the cleavage of Notch by GSEC. In 2020, MD simulations were performed to compare the binding of Notch and APP C83 to GSEC (Dehury et al., 2020b). The simulations revealed distinct conformational states of PS1 helices and thermal β-strand-to-transitions of C83 and Notch, as well as distinct hydrogen bond dynamics and water accessibility of the PS1 catalytic site. The "RKRR" motif contributes significantly to Notch binding and serves as a "membrane anchor" that prevents Notch displacement. The two substrates induce different conformational states of PS1, with Notch mostly present in a closed state with shorter catalytic Asp distance. This was suggested to result in different outcomes of Notch and APP cleavage, as the latter appears more imprecise in initial ε cleavage (Dehury et al., 2020b).

In 2022, MD simulations started from the cryo-EM structure of Notch1-bound GSEC (PDB: 6IDF) could not successfully restore the hydrogen bond between the catalytic Asp of PS1 and residue G1753 of Notch1 that corresponds to the initial cleavage site (Chen and Zacharias, 2022). Then comparative homology modeling using MODELLER was applied to reconstruct the Notch1-bound GSEC using the C83-bound complex structure as a template. MD simulations of the homology model correctly captured the catalytic geometry at the expected substrate residue. Further MD simulations revealed an internal docking site located close to the region where the helical conformation of the substrates is interrupted and continues toward the active site in an extended conformation. This site consists of two non-polar

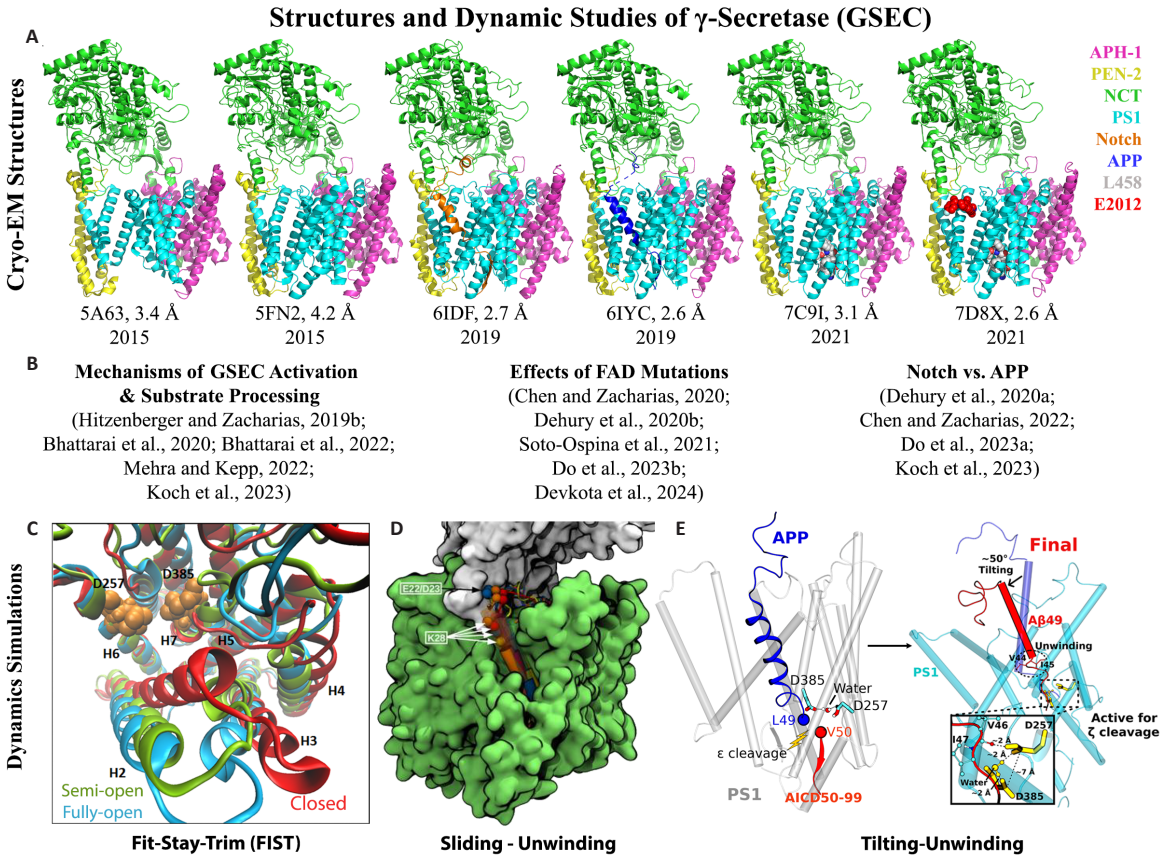

Figure 1 | **Advances in structural and dynamic studies of GSEC.**
(A) Representative cryo-EM structures of apo GSEC (PDB: 5A63, 2015), DAPT-bound GSEC (PDB: 5FN2, 2015), Notch-bound GSEC (PDB: 6IDF, 2019), APP-bound GSEC (PDB: 6IYC, 2019), inhibitor L458-bound GSEC (PDB: 7C9I, 2021) and both inhibitor L458 and modulator E2012-bound GSEC (PDB: 7D8X, 2021). The four protein subunits of GSEC are shown in ribbons, including APH-1 (purple), PEN-2 (yellow), NCT (green), and PS1 (cyan), similarly for the peptide substrates such as Notch (orange) and APP (blue). The small molecules are represented by spheres with the carbon atoms of L458 colored in gray and E2012 in red. PyMOL was used to prepare the structural images. Note that the other PDB structures of GSEC including 5FN3, 5FN4, 5FN5, 6LR4, and 7C9I are not shown here. (B) Dynamics simulation studies published after 2020 that mainly address the mechanisms of GSEC activation and substrate processing, effects of FAD mutations, and comparison of Notch and APP binding and proteolysis in GSEC. (C) Distinct conformational states identified from MD simulations of GSEC, including the "Closed", "Semi-open" and "Fully-open", leading to the "Fit-Stay-Trim (FIST)" mechanism suggested for substrate processing by GSEC. Adapted with permission from Somavarapu and Kepp (2017). (D) The "Sliding-Unwinding" mechanism suggested from MD simulations. Adapted with permission from Hitzenberger and Zacharias (2019c). (E) The "Tilting-Unwinding" mechanism suggested from GaMD simulations of tripeptide trimming of $A\beta_{49}$ after the ε-cleavage of APP. Adapted with permission from Bhattarai et al. (2022). APH-1: Anterior pharynx-defective 1; APP: amyloid precursor protein; GSEC : γ-secretase; NCT: nicastrin; PEN-2: presenilin enhancer 2; PS1: presenilin.

pockets preferentially filled with large hydrophobic or aromatic substrate residues. Simulations on a K28A mutation of APP indicated that the internal docking site opposes the tendency of substrate dissociation due to a hydrophobic mismatch at the membrane boundary. The hydrophobic mismatch is caused by the K28 residue in APP during processing and substrate movement toward the enzyme active site (Chen and Zacharias, 2022).

The incorrect registry of the Notch1 substrate in the cryo-EM structure of Notch1-bound GSEC was also identified in GaMD simulations (Do et al., 2023a). We prepared simulation systems starting from the cryo-EM structure of Notch1-bound GSEC and failed to capture the proper cleavages of wildtype and L36F Notch by GSEC. According to the structural alignment of the cryo-EM structures, the Notch1 substrate in the 6IDF PDB structure was found to be shifted one residue upwards compared with APP, the latter of which had been demonstrated to be positioned for proper cleavage by GSEC. Then every residue of APP substrate was systematically mutated to the corresponding Notch residue to prepare a resolved model of Notch-bound GSEC complexes. GaMD simulations of the resolved model successfully captured GSEC activation for proper cleavages of both wildtype and L36F mutant Notch., being highly consistent with the mass spectrometry experimental findings. The computational amino acid replacement method could be potentially applied to other GSEC substrates, for which the initial cleavage site is known but with no cryo-EM structure of the substrate-bound GSEC. This could allow MD simulations to decipher mechanisms of GSEC activation for the cleavage of various substrates (Do et al., 2023a).

Controversial Effects of Familial Alzheimer's Disease Mutations

All-atom MD simulations have been applied to investigate the effects of five prominent FAD mutations (E280A, G384A, A434C, and L435F) of PS1 and the V717I mutation of APP, suggesting that FAD mutations destabilize the enzyme-substrate complexes (Dehury et al., 2020a). Note that both catalytic aspartates were deprotonated in the setup of these simulation systems, likely resulting in repulsion between the negative charges, and GSEC could thus not become active for substrate proteolysis during the simulations. Nevertheless, analysis of the distance from catalytic aspartates to substrate

cleavage sites showed that the FAD mutations tend to increase the space and variability in the substrate-binding site during MD simulations. It was suggested that FAD mutations favor "looser" substrate binding to GSEC, causing "imprecise cleavage" of the APP substrate (Dehury et al., 2020a). This was in line with experimental findings that AD-causing mutations destabilize the GSEC-APP/Aβn interactions and enhance the production of longer Aβs (Petit et al., 2019; Szaruga et al., 2021).

Umbrella sampling MD simulations were performed to calculate free energy profiles of PS1 FAD mutants (including L250S, S390I, L392V, L435S, P436S, and I439V) and the wildtype of the substrate-free (apo) GSEC complex, using the distance between the Cγ atoms of the two catalytic aspartates as the reaction coordinate (Chen and Zacharias, 2020). Two low-energy conformational states were identified from the calculated free energy profiles, i.e., a "closed" state with a direct contact between the catalytic aspartates and an "open" water-bridged state that is active for proteolysis. FAD mutations could significantly modulate the free energy difference between the closed and open states. For FAD mutations with reduced experimental enzyme activity, an increased penalty was found for the closed to open (active) state transitions. Among the studied FAD mutations, only two were located at the active site. Therefore, the simulation findings suggested that the modulation of the closed/open equilibrium and perturbation of the open (active) catalytic geometry could be possible mechanisms of how FAD mutations affect GSEC activity. However, these simulations were carried out for the apo GSEC and the effects of FAD mutations on enzyme-substrate interactions could not be explored.

More recently, we combined GaMD simulations with complementary experiments to determine the effects of six representative PS1 FAD mutations (P117L, I143T, L166P, G384A, L435F, and L286V) in the enzyme-substrate interactions and processive proteolysis of APP by GSEC (Devkota et al., 2024; Do et al., 2023b). Western blotting and mass spectrometry experiments showed that all six PS1 FAD mutations rendered GSEC less active for the ε cleavage of APP. Multiple distinct low-energy conformational states were identified from the free energy profiles calculated from GaMD simulations of the wildtype and PS1 FAD-mutant of APP-bound GSEC, including the "Active", "Inhibited" and "Intermediate I1-I5". The P117L and L286V FAD mutants could still sample the "Active" state for substrate cleavage, but with noticeably reduced conformational space compared with the wild type. The other mutants hardly visited the "Active" state. The PS1 FAD mutants were found to reduce GSEC proteolytic activity by hindering APP residue L49 from proper orientation in the active site and/or disrupting the distance between the catalytic aspartates (Do et al., 2023b). Furthermore, the FAD mutations were found to reduce the flexibility of GSEC during the ε cleavage and tripeptide trimming of APP during the GaMD simulations. The simulation findings were supported by fluorescence lifetime imaging microscopy in cultured cells, showing stabilization by FAD mutations of enzyme-substrate and/or enzyme-intermediate complexes (Devkota et al., 2024). Neuronal expression of C99 and/or PS1 in *Caenorabditis elegans* led to age-dependent synaptic loss only when one of the transgenes carried an FAD mutation. Designed mutations that stabilize the enzyme-substrate complex and block proteolysis likewise led to synaptic loss. Therefore, in contrast to earlier models (Petit et al., 2019; Dehury et al., 2020a; Szaruga et al., 2021), our GaMD simulations and biochemical, biophysical, and animal model experiments collectively suggested that FAD mutations stabilize the GSEC-APP/Aβ complexes and these "stalled complexes" may be the trigger of FAD pathogenesis (Devkota et al., 2024).

Conclusion

Remarkable advances have been recently made in structural and dynamics simulation studies of GSEC. Different mechanisms have been proposed for substrate proteolysis of APP by GSEC, including the "Fit-Stay-Trim", "Sliding-Unwinding" and "Tilting-Unwinding" mechanisms (**Figure 1**). Notably, the "Fit-Stay-Trim" mechanism was originally proposed based on MD simulations of apo GSEC with both catalytic aspartates deprotonated (Somavarapu and Kepp, 2017). The model could potentially be refined with direct simulations of the substrate-bound GSEC, for which only one of the two catalytic aspartates should be deprotonated for proper proteolysis. The "Sliding-Unwinding" mechanism was derived based on restraint MD simulations, in which the N-terminus of the Aβ peptide was anchored to maintain its interaction with PS1 (Hitzenberger and Zacharias, 2019c). More recent GaMD simulations achieved unconstrained enhanced sampling of the GSEC-APP/$Aβ_{49}$ interactions, which suggested a different "Tilting-Unwinding" mechanism for substrate proteolysis by GSEC (Bhattarai et al., 2020, 2022). However, all other cleavage steps of APP in the two ($Aβ_{42}$ and $Aβ_{40}$) pathways would need to be simulated in future studies to confirm the mechanistic model.

In addition, the exact pathways of substrate binding to GSEC and the release of proteolysis products from GSEC remain elusive (Hitzenberger et al., 2020). Since these slow processes are far beyond the reach of conventional atomistic MD simulations, they present challenging tasks for computational modeling. However, they are critically important problems for future studies to fully understand the functional mechanisms of GSEC.

Finally, MD simulations have correctly predicted the target binding sites of the GSI (Hitzenberger and Zacharias, 2019a) and GSM (Mehra and Kepp, 2021), which were largely confirmed by subsequent cryo-EM structures (Yang et al., 2021). While GSIs could cause adverse side effects, the GSMs provide more promising selective therapeutic treatment of AD. The dynamic effects of the GSMs on both the wildtype and FAD mutations of the GSEC-APP/Aβ complexes need to be examined in future studies. Moreover, it is critical to validate *in silico* findings (e.g., MD simulations) in the *in vitro* and *in vivo* experiments including western blotting, mass spectrometry, cellular assays, and animal models. In this way, we shall understand the mechanism of allosteric modulation of substrate processing in GSEC to rationally design more effective GSMs for AD.

Author contributions: *Manuscript conception and design, data collection, analysis, and interpretation: YM; manuscript writing, approval of the final version of the manuscript: YM and MSW.*
Conflicts of interest: *The authors declare no conflicts of interest.*
Data availability statement: *Not applicable.*

References

Aguayo-Ortiz R, Chavez-Garcia C, Straub JE, Dominguez L (2017) Characterizing the structural ensemble of gamma-secretase using a multiscale molecular dynamics approach. Chem Sci 8:5576-5584.
Bai XC, Rajendra E, Yang G, Shi Y, Scheres SH (2015a) Sampling the conformational space of the catalytic subunit of human gamma-secretase. Elife 4:e11182.
Bai XC, Yan CY, Yang GH, Lu PL, Ma D, Sun LF, Zhou R, Scheres SHW, Shi YG (2015b) An atomic structure of human gamma-secretase. Nature 525:212-217.
Bhattarai A, Devkota S, Bhattarai S, Wolfe MS, Miao Y (2020) Mechanisms of γ-secretase activation and substrate processing. ACS Cent Sci 6:969-983.
Bhattarai A, Devkota S, Do HN, Wang J, Bhattarai S, Wolfe MS, Miao Y (2022) Mechanism of tripeptide trimming of amyloid β-peptide 49 by γ-secretase. J Am Chem Soc 144:6215-6226.
Bolduc DM, Montagna DR, Seghers MC, Wolfe MS, Selkoe DJ (2016) The amyloid-beta forming tripeptide cleavage mechanism of gamma-secretase. Elife 5:e17578.
Bursavich MG, Harrison BA, Blain JF (2016) Gamma secretase modulators: new Alzheimer's drugs on the horizon? J Med Chem 59:7389-7409.
Chen SY, Zacharias M (2020) How mutations perturb γ-secretase active site studied by free energy simulations. ACS Chemical Neuroscience 11:3321-3332.
Chen SY, Zacharias M (2022) An internal docking site stabilizes substrate binding to γ-secretase: analysis by molecular dynamics simulations. Biophys J 121:2330-2344.
Coric V, Salloway S, van Dyck CH, Dubois B, Andreasen N, Brody M, Curtis C, Soininen H, Thein S, Shiovitz T, Pilcher G, Ferris S, Colby S, Kerselaers W, Dockens R, Soares H, Kaplita S, Luo F, Pachai C, Bracoud L, et al. (2015) Targeting prodromal Alzheimer disease with avagacestat: a randomized clinical trial. JAMA neurology 72:1324-1333.
Dehury B, Tang N, Kepp KP (2019a) Molecular dynamics of C99-bound γ-secretase reveal two binding modes with distinct compactness, stability, and active-site retention: implications for Aβ production. Biochem J 476:1173-1189.
Dehury B, Tang N, Blundell TL, Kepp KP (2019b) Structure and dynamics of gamma-secretase with presenilin 2 compared to presenilin 1. RSC Adv 9:20901-20916.
Dehury B, Somavarapu AK, Kepp KP (2020a) A computer-simulated mechanism of familial Alzheimer's disease: mutations enhance thermal dynamics and favor looser substrate-binding to gamma-secretase. J Struct Biol 212:107648.
Dehury B, Tang N, Mehra R, Blundell TL, Kepp KP (2020b) Side-by-side comparison of Notch- and C83 binding to gamma-secretase in a complete membrane model at physiological temperature. RSC Adv 10:31215-31232.
Dehury B, Kepp KP (2021) Membrane dynamics of gamma-secretase with the anterior pharynx-defective 1B subunit. J Cell Biochem 122:69-85.
Devkota S, Zhou R, Nagarajan V, Maesako M, Do H, Noorani A, Overmeyer C, Bhattarai S, Douglas JT, Saraf A, Miao Y, Ackley BD, Shi Y, Wolfe MS (2024) Familial Alzheimer mutations stabilize synaptotoxic γ-secretase-substrate complexes. Cell Rep doi:10.1016/j.celrep.2024.113761.
Do HN, Malvankar SR, Wolfe MS, Miao Y (2023a) Molecular Dynamics Activation of γ-Secretase for Cleavage of Notch1 Substrate. ACS Chem Neurosci 14:4216-4226.
Do HN, Devkota S, Bhattarai A, Wolfe MS, Miao Y (2023b) Effects of presenilin-1 familial Alzheimer's disease mutations on γ-secretase activation for cleavage of amyloid precursor protein. Commun Biol 6:174.
Doody RS, Raman R, Farlow M, Iwatsubo T, Vellas B, Joffe S, Kieburtz K, He F, Sun XY, Thomas RG, Aisen PS, Siemers E, Sethuraman G, Mohs R (2013a) A phase 3 trial of semagacestat for treatment of Alzheimer's disease. N Engl J Med 369:341-350.
Doody RS, Raman R, Farlow M, Iwatsubo T, Vellas B, Joffe S, Kieburtz K, He F, Sun X, Thomas RG, Aisen PS, Siemers E, Sethuraman G, Mohs R (2013b) A phase 3 trial of semagacestat for treatment of Alzheimer's disease. N Engl J Med 369:341-350.
Gertsik N, Ende CWA, Geoghegan KF, Nguyen C, Mukherjee P, Mente S, Seneviratne U, Johnson DS, Li YM (2017) Mapping the binding site of BMS-708163 on gamma-secretase with cleavable photoprobes. Cell Chem Biol 24:3-8.
Götz A, Högel P, Silber M, Chaitoglou I, Luy B, Muhle-Goll C, Scharnagl C, Langosch D (2019a) Increased H-bond stability relates to altered ε-cleavage efficiency and Aβ levels in the I45T familial Alzheimer's disease mutant of APP. Sci Rep 9:5321.
Götz A, Mylonas N, Högel P, Silber M, Heinel H, Menig S, Vogel A, Feyrer H, Huster D, Luy B (2019b) Modulating hinge flexibility in the APP transmembrane domain alters γ-secretase cleavage. Biophys J 116:2103-2120.
Green RC, Schneider LS, Amato DA, Beelen AP, Wilcock G, Swabb EA, Zavitz KH, Tarenflurbil Phase 3 Study G (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.
Gu Y, Misonou H, Sato T, Dohmae N, Takio K, Ihara Y (2001) Distinct intramembrane cleavage of the beta-amyloid precursor protein family resembling gamma-secretase-like cleavage of Notch. J Biol Chem 276:35235-35238.
Haass C, Kaether C, Thinakaran G, Sisodia S (2012) Trafficking and proteolytic processing of APP. Cold Spring Harb Perspect Med 2:a006270.
Hitzenberger M, Zacharias M (2019a) Uncovering the binding mode of gamma-secretase inhibitors. ACS Chem Neurosci 10:3398-3403.
Hitzenberger M, Zacharias M (2019b) Gamma-secretase studied by atomistic molecular dynamics simulations: global dynamics, enzyme activation, water distribution and lipid binding. Front Chem 6:640.
Hitzenberger M, Zacharias M (2019c) Structural modeling of gamma-secretase A beta(n) complex formation and substrate processing. Acs Chemical Neuroscience 10:1826-1840.
Hitzenberger M, Gotz A, Menig S, Brunschweiger B, Zacharias M, Scharnagl C (2020) The dynamics of gamma-secretase and its substrates. Semin Cell Dev Biol 105:86-101.
Karplus M, McCammon JA (2002) Molecular dynamics simulations of biomolecules. Nat Struct Biol 9:646-652.
Koch M, Enzlein T, Chen SY, Petit D, Lismont S, Zacharias M, Hopf C, Chávez-Gutiérrez L (2023) APP substrate ectodomain defines Aβ length by restraining γ-secretase processivity and facilitating product release. EMBO J 42:e114372.
Kong R, Chang S, Xia W, Wong ST (2015) Molecular dynamics simulation study reveals potential substrate entry path into gamma-secretase/presenilin-1. J Struct Biol 191:120-129.
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.
Lee JY, Feng Z, Xie XQ, Bahar I (2017) Allosteric modulation of intact gamma-secretase structural dynamics. Biophys J 113:2634-2649.
Lu PL, Bai XC, Ma D, Xie T, Yan CY, Sun LF, Yang GH, Zhao YY, Zhou R, Scheres SHW, Shi YG (2014) Three-dimensional structure of human gamma-secretase. Nature 512:166-170.
Luo JE, Li YM (2022) Turning the tide on Alzheimer's disease: modulation of gamma-secretase. Cell Biosci 12:2.
Mehra R, Kepp KP (2021) Computational prediction and molecular mechanism of gamma-secretase modulators. Eur J Pharm Sci 157:105626.
Mehra R, Kepp KP (2022) Understanding familial Alzheimer's disease: the fit-stay-trim mechanism of γ-secretase. WIREs Comput Mol Sci 12:e1556.
Northrop DB (2001) Follow the protons: a low-barrier hydrogen bond unifies the mechanisms of the aspartic proteases. Acc Chem Res 34:790-797.
Petit D, Hitzenberger M, Lismont S, Zoltowska KM, Ryan NS, Mercken M, Bischoff F, Zacharias M, Chávez-Gutiérrez L (2019) Extracellular interface between APP and Nicastrin regulates A, Zacharias M, Chávezs. Eur J Pharm Sci 157:ors. EMBO J 38:e101494.
Qi-Takahara Y, Morishima-Kawashima M, Tanimura Y, Dolios G, Hirotani N, 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 gamma-secretase. J Neurosci 25:436-445.
Sato T, Dohmae N, Qi Y, Kakuda N, Misonou H, Mitsumori R, Maruyama H, Koo EH, Haass C, Takio K, Morishima-Kawashima M, Ishiura S, Ihara Y (2003) Potential link between amyloid beta-protein 42 and C-terminal fragment gamma 49-99 of beta-amyloid precursor protein. J Biol Chem 278:24294-24301.
Selkoe DJ, Hardy J (2016) The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO Mol Med 8:595-608.
Somavarapu AK, Kepp KP (2016) The dynamic mechanism of presenilin-1 function: Sensitive gate dynamics and loop unplugging control protein access. Neurobiol Dis 89:147-156.
Somavarapu AK, Kepp KP (2017) Membrane dynamics of γ-secretase provides a molecular basis for β-amyloid binding and processing. ACS Chem Neurosci 8:2424-2436.
Soto-Ospina A, Araque Marín P, Bedoya G, Sepulveda-Falla D, Villegas Lanau A (2021) Protein predictive modeling and simulation of mutations of presenilin-1 familial Alzheimer's disease on the orthosteric site. Front Mol Biosci 8:387.
Steiner H, Fukumori A, Tagami S, Okochi M (2018) Making the final cut: pathogenic amyloid-beta peptide generation by gamma-secretase. Cell Stress 2:292-310.
Szaruga M, Munteanu B, Lismont S, Veugelen S, Horre K, Mercken M, Saido TC, Ryan NS, De Vos T, Savvides SN, Gallardo R, Schymkowitz J, Rousseau F, Fox NC, Hopf C, De Strooper B, Chavez-Gutierrez L (2021) Alzheimer's-causing mutations shift Abeta length by destabilizing gamma-secretase-Abetan interactions. Cell 184:2257-2258.
Takami M, Nagashima Y, Sano Y, Ishihara S, Morishima-Kawashima M, Funamoto S, Ihara Y (2009) Gamma-secretase: successive tripeptide and tetrapeptide release from the transmembrane domain of beta-carboxyl terminal fragment. J Neurosci 29:13042-13052.
Weggen S, Eriksen JL, Das P, Sagi SA, Wang R, Pietrzik CU, Findlay KA, Smith TE, Murphy MP, Butler T, Kang DE, Marquez-Sterling N, Golde TE, Koo EH (2001) A subset of NSAIDs lower amyloidogenic A beta 42 independently of cyclooxygenase activity. Nature 414:212-216.
Wilcock GK, Black SE, Hendrix SB, Zavitz KH, Swabb EA, Laughlin MA, Tarenflurbil Phase IISi (2008) Efficacy and safety of tarenflurbil in mild to moderate Alzheimer's disease: a randomised phase II trial. Lancet Neurol 7:483-493.
Wolfe MS (2007) Gamma-secretase modulators. Curr Alzheimer Res 4:571-573.
Wolfe MS (2009) Intramembrane proteolysis. Chem Rev 109:1599-1612.
Yang G, Zhou R, Guo X, Yan C, Lei J, Shi Y (2021) Structural basis of gamma-secretase inhibition and modulation by small molecule drugs. Cell 184:521-533.
Yang GH, Zhou R, Zhou Q, Guo XF, Yan CY, Ke M, Lei JL, Shi YG (2019) Structural basis of Notch recognition by human gamma-secretase. Nature 565:192-197.
Zhou R, Yang GH, Guo XF, Zhou Q, Lei JL, Shi YG (2019) Recognition of the amyloid precursor protein by human gamma-secretase. Science 363:708-715.

C-Editors: Zhao M, Liu WJ, Qiu Y; T-Editor: Jia Y