

Complex Relationships between Substrate Sequence and Sensitivity to Alterations in γ -Secretase Processivity Induced by γ -Secretase Modulators

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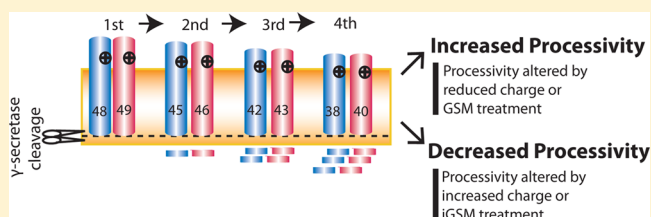
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Supporting Information

ABSTRACT: γ -Secretase catalyzes the final cleavage of the amyloid precursor protein (APP), resulting in the production of amyloid- β ($A\beta$) peptides with different carboxyl termini. Presenilin (PSEN) and amyloid precursor protein (APP) mutations linked to early onset familial Alzheimer's disease modify the profile of $A\beta$ isoforms generated, by altering both the initial γ -secretase cleavage site and subsequent processivity in a manner that leads to increased levels of the more amyloidogenic $A\beta_{42}$ and in some circumstances $A\beta_{43}$. Compounds termed γ -secretase modulators (GSMs) and inverse GSMs (iGSMs) can decrease and increase levels of $A\beta_{42}$, respectively. As GSMs lower the level of production of pathogenic forms of long $A\beta$ isoforms, they are of great interest as potential Alzheimer's disease therapeutics. The factors that regulate GSM modulation are not fully understood; however, there is a growing body of evidence that supports the hypothesis that GSM activity is influenced by the amino acid sequence of the γ -secretase substrate. We have evaluated whether mutations near the luminal border of the transmembrane domain (TMD) of APP alter the ability of both acidic, nonsteroidal anti-inflammatory drug-derived carboxylate and nonacidic, phenylimidazole-derived classes of GSMs and iGSMs to modulate γ -secretase cleavage. Our data show that point mutations can dramatically reduce the sensitivity to modulation of cleavage by GSMs but have weaker effects on iGSM activity. These studies support the concept that the effect of GSMs may be substrate selective; for APP, it is dependent on the amino acid sequence of the substrate near the junction of the extracellular domain and luminal segment of the TMD.



Abnormal metabolism of $A\beta$ that promotes its aggregation and accumulation in the brain is tightly linked to the development of Alzheimer's disease (AD).¹ $A\beta$ is an ~ 4 kDa peptide that is derived from the amyloid precursor protein (APP) through a series of sequential enzymatic reactions involving β - and γ -secretase, respectively.² The ectodomain of APP is cleaved by β -secretase, releasing soluble APP β (sAPP β) and generating a 99-amino acid β -carboxyl-terminal membrane fragment (CTF β or C99). CTF β is then sequentially cleaved within the membrane-spanning domain by γ -secretase to produce the APP intracellular domain (AICD) and the various $A\beta$ isoforms. Genetic, biochemical, animal modeling, and pathological studies strongly suggest that $A\beta_{x-42}$ is the pathogenic form of $A\beta$. The vast majority of APP and PSEN mutations linked to early onset familial Alzheimer's disease (FAD) results in increased levels of $A\beta_{42}$.³ $A\beta_{1-42}$ aggregates faster *in vitro* than $A\beta_{1-40}$.⁴ In transgenic modeling studies, $A\beta_{42}$ but not $A\beta_{40}$ seeds deposition *in vivo*.⁵ Further, $A\beta_{40}$ may protect transgenic mice from amyloid deposition.^{6,7} Moreover, $A\beta_{x-42}$ is typically the earliest detectable form of

$A\beta$ in the AD brain^{8,9} and the species that is most consistently detected in AD brain.⁸⁻¹¹ Therefore, given that small increases in the levels of long $A\beta$ isoforms ($A\beta_{42}$ and possibly $A\beta_{43}$) appear to be capable of prompting the aggregation and accumulation of $A\beta$, triggering a complex pathological cascade leading to AD, lowering the levels of these longer forms of $A\beta$ is still considered a potential prophylactic approach to AD therapy.¹²⁻¹⁴

γ -Secretase modulators (GSMs) are small molecules that lower $A\beta_{42}$ levels by altering γ -secretase processivity without significantly altering the initial ϵ -cleavage of APP.^{15,16} Two major classes of GSMs have been identified and intensively investigated.¹⁷ Acidic GSMs, which include nonsteroidal anti-inflammatory drug (NSAID) and NSAID-like compounds, contain a carboxylic acid group that is anchored to various hydrophilic scaffolds.¹⁸ Nonacidic GSMs have largely been

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based on a piperazinyl pyrimidine.^{19,20} Acidic GSMs characteristically shift γ -secretase cleavage by decreasing the level of $A\beta$ 42 and concomitantly increasing the level of $A\beta$ 38, whereas nonacidic GSMs decrease levels of both $A\beta$ 40 and $A\beta$ 42 while increasing levels of $A\beta$ 37 and $A\beta$ 38. More recently, a distinct class of triterpenoid nonacidic GSMs that lower the levels of both $A\beta$ 42 and $A\beta$ 38 have been identified, while sparing the levels of $A\beta$ 40 and total $A\beta$.²¹ In addition, other compounds that selectively increase the level of $A\beta$ 42 and decrease the level of $A\beta$ 38 have been identified and are termed inverse GSMs (iGSMs).²²

γ -Secretase is a multiunit aspartyl protease known to cleave multiple type 1 membrane proteins within their TMDs.²³ Presenilins 1 and 2 (PSEN1 and PSEN2, respectively) are the catalytic subunits of γ -secretase, and anterior pharynx-defective 1 (APH-1), nicastrin, and presenilin enhancer protein 2 (PEN-2) are additional subunits required for γ -secretase complex formation and activity.²⁴ γ -Secretase exhibits little cleavage specificity and appears to preferentially cleave the substrate's transmembrane "stubs" that have been generated following sheddase cleavage that removes the majority of the substrate's ectodomain. It is also thought that colocalization of substrate and γ -secretase within membrane microdomains may play a critical role in regulating cleavage.^{25,26} γ -Secretase cleavage can play an essential role in transmembrane signaling²⁷ or termination of transmembrane signaling,²⁸ but for many substrates, it may play a role akin to that of the proteasome by recycling these membrane protein stubs.^{29,30}

γ -Secretase cleavage of APP and Notch-1 has been studied more intensively than cleavage of other substrates.^{31–35} A stepwise cleavage model, originally proposed by Takami et al., has provided valuable insights into how a single enzymatic activity generates a spectrum of $A\beta$ peptides.³⁴ In this model, γ -secretase initially cleaves APP at one of several potential ϵ -sites within the APP TMD but near the cytoplasmic face of the membrane. Subsequently, there is successive stepwise cleavage of the substrate. Depending on the number of stepwise cleavages, typically three to five, multiple different $A\beta$ peptides can be produced. For APP, there is evidence that the initial ϵ -cleavage can occur at least at three sites, and that differential ϵ -cleavage results in preferential processing along specific product lines.³⁸ Thus, initial ϵ -cleavage generating $A\beta$ 48 or -51 preferentially leads to $A\beta$ 42 production, and ϵ -cleavage at $A\beta$ 49 preferentially leads to $A\beta$ 40 production. Notably, elegant studies from several groups show that APP and PS1 mutations linked to AD may have effects on the rate of the initial ϵ -cleavage. However, through a combination of decreasing subsequent cleavage processivity, altering the site of the initial ϵ -cleavage to favor the $A\beta$ 42 product line, or some combination, these effects can increase the relative level of production of $A\beta$ 42.^{31,35,37} Although this is a useful model, it is clear that the stepwise processing is quite complex. Indeed, more recent studies suggest that (i) the product lines are not invariant, (ii) additional cleavages besides tri- and tetrapeptide removal can occur, and (iii) more physiologic systems show more heterogeneity with respect to processivity.^{38,39}

Previous site-directed mutagenesis studies of APP have identified key amino acid changes within the APP CTF β that can dramatically alter the $A\beta$ species produced by γ -secretase cleavage.^{32,33,40–42} These data reveal that mutations of the lysine residue that delineates the ectodomain of APP from its TMD (K624 based on the APP695 isoform)^{20,33,41} and the GXXXG motifs immediately downstream of that lysine^{20,43}

have dramatic effects on $A\beta$ peptide profiles. Although not all of these mutations were used for examination of cleavage at the ϵ -site, those that did showed no effects on ϵ -site utilization.³³ This finding suggests that these mutations likely cause shifts in $A\beta$ peptides by altering γ -secretase processivity. As the NSAID-based GSMs require a carboxylic acid for GSM activity⁴⁴ and can often be converted to iGSMs by modifications of the acid group to either neutral or positively charged groups,^{22,45} we hypothesized that the carboxylic acid of acidic GSMs, some of which can bind to the substrate in the GXXXG motif,^{46,47} might interact with and neutralize the charge at K624 and enhance cleavage processivity, resulting in a decreased level of $A\beta$ 42. Conversely, we predicted that nonacidic GSMs would not interact with this residue. To test this hypothesis, we studied the effects of GSMs on previously studied and novel mutations in APP that altered K624 or modified the charge of adjacent amino acids. Data generated from these studies show that in a manner independent of the class of GSM, several of these mutations have dramatic effects on the sensitivity of the mutant substrate to cleavage modulation. In contrast, iGSMs are still able to modulate cleavage of most mutant substrates. Although these data do not support a model in which acidic and nonacidic GSMs have a differential effect on processivity that is mediated by charged residues at the end of the substrate's ectodomain, they indicate that both classes of GSMs have highly substrate selective effects.

■ EXPERIMENTAL PROCEDURES

Mutagenesis and Expression Plasmid Construction.

Point mutations were generated at or near lysine 624 in pAG3-APP695wt [APP695 K624R, N623K, G625K, G625K/A626K (3xK), K624E, and K624E/G625E/A626E (3xE)] using polymerase chain reaction mutagenesis;⁴⁸ all sequences were verified by DNA sequencing.⁴⁹ The $A\beta$ peptides generated from the various APP mutants are numbered with respect to the first N-terminal residue (Asp-1) of the $A\beta$ peptide.⁵⁰

Cell Culture and Transfection. Chinese hamster ovary (CHO) cells were grown in Ham's F-12 medium (Life Technologies) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO₂ in six-well tissue culture plates (Costar). Cells were transfected with the DNA plasmids using polyethylenimine reagent as described previously.⁵¹

Compounds. GSM-1⁴² and Cmpd2¹⁹ were synthesized by A. Fauq at the Mayo Clinic Chemical Core (Jacksonville, FL). Fenofibrate was purchased from Sigma-Aldrich. All compound stocks were prepared in dimethyl sulfoxide (DMSO) to final concentrations of 10–30 mM. Compounds were tested on CHO cells stably overexpressing the various APP mutants. For compound testing, the cells were incubated for 16 h in the presence of the compound prepared in OptiMEM reduced serum medium (Life Technologies, Inc., Carlsbad, CA) containing 1% fetal bovine serum. Unless otherwise specified, DMSO was used as the vehicle control.

Mass Spectrometry of $A\beta$. For matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analysis of $A\beta$ peptides, a GSM or inverse GSM compound was added to the CHO cells expressing one of the APP mutant forms described. Secreted $A\beta$ peptides from conditioned media were analyzed as previously described^{52,53} with the following modifications. Briefly, the mutant peptides were immunoprecipitated using Ab5 recognizing the $A\beta$ 1–16

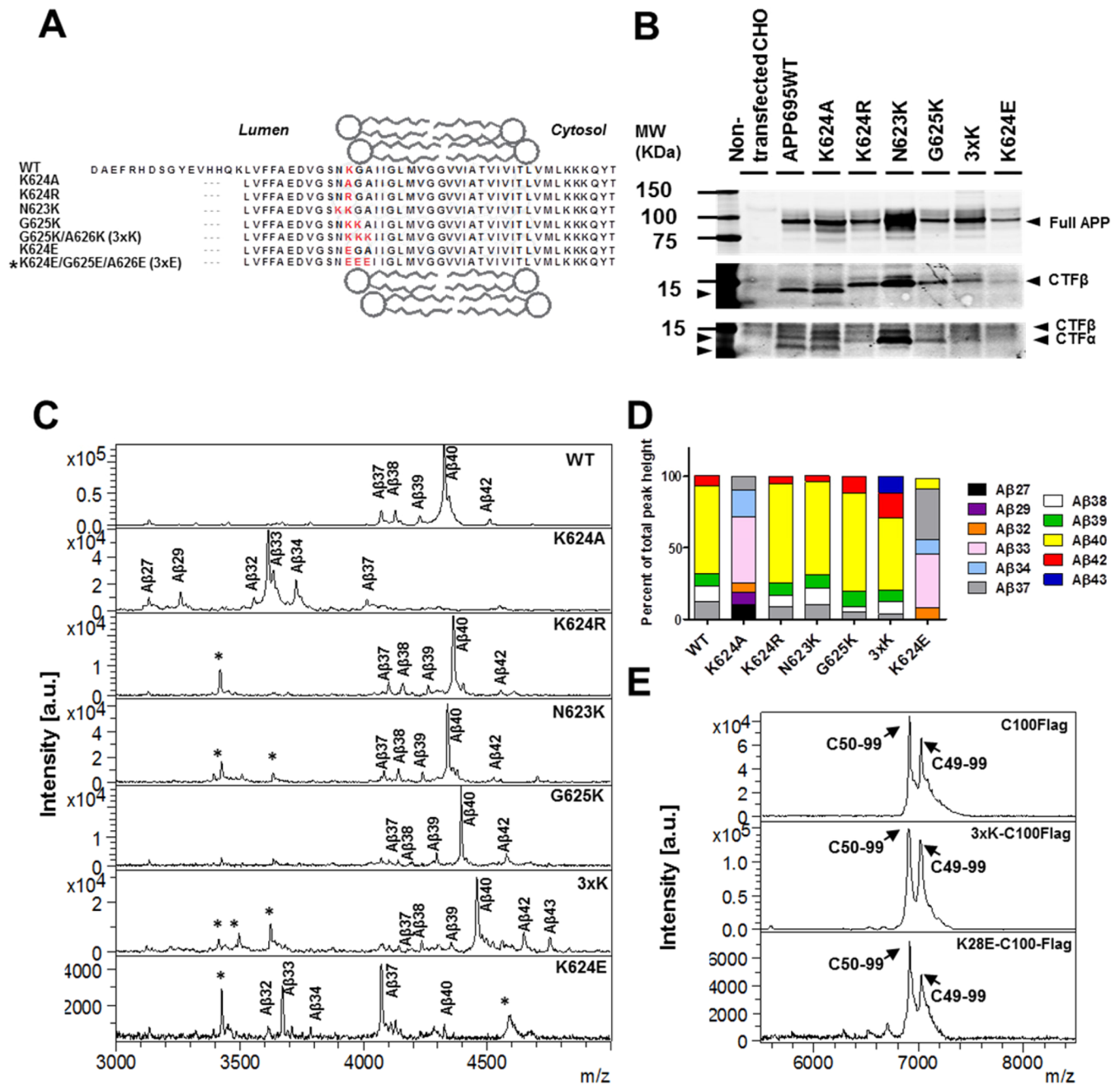


Figure 1. Effects of point mutations in APP and CTF on the production of Aβ and AICD, respectively. (A) The WT APP and mutant APP sequences examined in this study are highlighted. The 3xK construct has been tested only in the *in vitro* assay (marked with an asterisk). (Attempts to produce a stable cell line of the K624E/G625E/A626E construct were not successful.) (B) The WT APP and mutant APP were stably expressed in CHO cells and detected via Western blotting using a 6E10 monoclonal antibody.⁷⁴ (C) Aβ spectra obtained by MALDI-TOF analysis of conditioned media from CHO cells overexpressing WT and mutant forms of APP. Aβ isoforms are identified on the profiles with nonspecific peaks denoted with an asterisk. (D) Stacked bar graphs indicating the percent of each Aβ isoform derived from WT and mutant APP. These analyses were based on two to four experiments with two to five replicates in each experiment (the maximal SEM = ±2.5). (E) AICD spectra of WT CTFβ tagged with a Flag peptide (C100Flag), 3xK CTFβ tagged with Flag (3xK-C100Flag), and K28E CTFβ tagged with Flag (K28E-C100Flag) after immunoprecipitation and MALDI-TOF analysis. The positions of the two major products produced by γ-secretase cleavage at the ε-site, AICD50–99 and AICD49–99, are indicated. GSI treatment served as a control to select specific AICD peaks (data not shown).

epitope⁵⁴ and sheep anti-mouse IgG magnetic Dynabeads (catalog no. 11201D, Life Technologies) and eluted with 0.1% trifluoroacetic acid in water. Eluted samples were mixed in a 2:1 ratio with saturated α-cyano-4-hydroxycinnamic acid (CHCA) matrix (Sigma) in an acetonitrile/methanol mixture (60:40) and loaded onto a CHCA-pretreated MSP 96 target plate-polished steel (part no. 224989, Bruker, Billerica, MA). Samples

were analyzed using a Bruker Microflex LRF-MALDI-TOF mass spectrometer.

Modulation index calculation. The modulation index (MI) was calculated as previously described.⁵⁵ Briefly, the MI is determined by comparing the ratio of each peak to the sum of the total peaks and then calculating the difference between sum of the longer Aβ peptides (e.g., Aβ₄₂) and that of the shorter Aβ

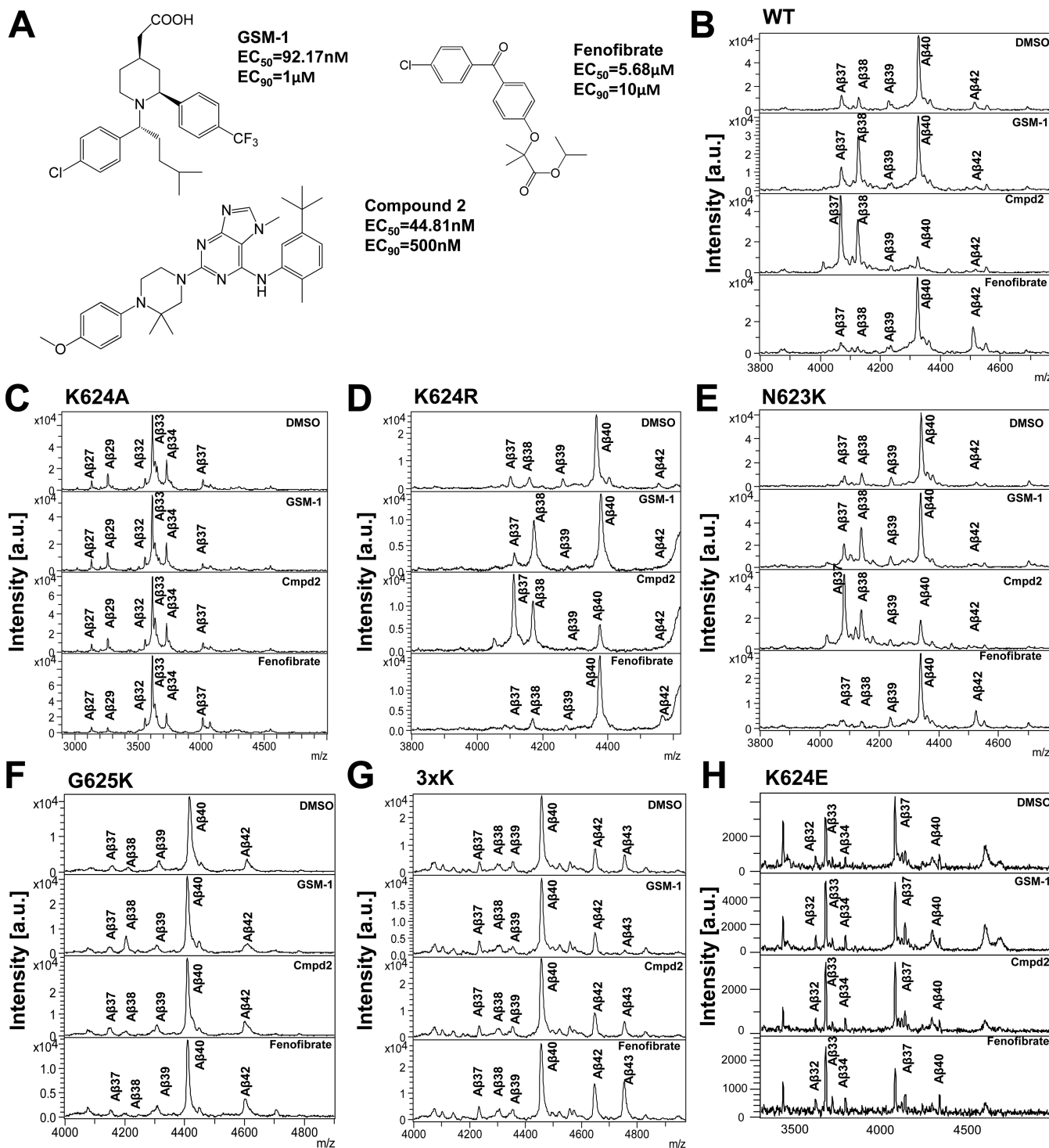


Figure 2. $A\beta$ profiles illustrated by MALDI-TOF analysis of the mutants after GSM and iGSM treatments. (A) Chemical structure of the acidic-type GSM (GSM-1), nonacidic-type GSM (Cmpd 2), and inverse GSM (fenofibrate) are shown. (B–G) $A\beta$ spectra from each mutant with or without drug treatments. Each $A\beta$ isoform is identified on the profile with nonspecific peaks marked with asterisks. DMSO treatment served as a control for all compounds. For each profile, the m/z range was adjusted to account for the molecular weight shift in the detected $A\beta$. The calculated and observed molecular weights of $A\beta$ peptides from each mutant are listed in Table 1 of the Supporting Information.

peptides (e.g., $A\beta_{37}$, $A\beta_{38}$, and $A\beta_{39}$). The result is subsequently normalized to the vehicle control which is arbitrarily set at zero. Using this method, a GSM will have a negative MI and an iGSM will have a positive MI, and compounds that shift cleavage to a greater extent will show a larger MI.⁵⁵

In Vitro γ -Secretase Assay for AICD Detection. DNA encoding C100 or C100 containing mutations was tagged with

Flag^{S6,S7} and cloned into pET-21b+ vectors (Life Technologies). The proteins were overexpressed and purified from *Escherichia coli* BL21 using a HiTrap Q-column (GE Life Science, Little Chalfont, U.K.). The membrane containing γ -secretase was isolated from the CHO S-1 cell line using sodium carbonate (100 mM, pH 11.0).⁵⁸ For the *in vitro* γ -secretase assay, each substrate at 25 μM was incubated with the

membrane (100 $\mu\text{g}/\text{mL}$) in 150 mM sodium citrate buffer (pH 6.8) containing complete protease inhibitor (Roche, Indianapolis, IN) for 2 h at 37 °C. The AICD fragments were captured using anti-Flag M2 magnetic beads (Sigma). The beads were then washed with water and the fragments eluted using a 10 μM solution of 0.1% TFA (Thermo Scientific) in water. The eluted fragments were further processed for mass spectrometry (MS), as described above.

Western Blotting. The WT- and mutant-expressing cells were harvested and lysed in radio-immunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate].⁵⁹ The lysates were subsequently used for immunoblotting and detection of full-length APP and carboxy-terminal fragments (CTFs). A β 1–16 monoclonal antibody 6E10 (1:1000) (Covance, Gaithersburg, MD) was used for APP and CTF β detection, and anti-APP-CT-20 (1:500) (Calbiochem) was used for CTF α/β detection. The blot was developed using an Odyssey infrared scanner (LiCor Biosciences, Lincoln, NE).

Statistical Analysis. *In vitro* data were expressed and graphed as means \pm the SEM using GraphPad Prism 5. Analysis was conducted by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc testing for group differences. The level of significance was set at $p < 0.05$ in all tests.

RESULTS

Effects of Point Mutations at or Adjacent to K624 in APP on A β Production. To evaluate the effects of positively charged residues at the interface of the APP ectodomain and its TMD on GSM and iGSM activity, we utilized a mutant APP695 construct that we had generated and characterized previously (APP-K624A)³³ and several newly generated constructs [APP695 K624R, N623K, G625K, G625K/A626K (termed 3xK), K624E, and K624E/G625E/A626E (termed 3xE)]. These constructs are schematically depicted in Figure 1A. Expression plasmids encoding these APP cDNAs were transfected into CHO cells and stable clones obtained for all except the 3xE construct. These stable lines overexpress APP and are processed into CTF β and CTF α ; however, the CTFs for K624R, N623K, G625K, 3xK, and K624E migrate more slowly than those derived from the WT and K624A APPs (Figure 1B). We performed immunoprecipitation followed by mass spectrometry (IP–MS) to assess A β production profiles from these transfected cells. Representative spectra from these experiments are shown in Figure 1C with the average percent of total peak height for each A β isoform obtained from multiple experiments graphically depicted in Figure 1D. As previously observed, the K624A mutant dramatically shifted the A β profile toward shorter peptides, with A β 1–33 and A β 1–34 being the major species detected. Compared to WT, the K624R and N623K mutants had a minimal effect on the A β profile. G625K and the 3xK constructs decreased the levels of A β 1–37 and A β 1–38 and increased the level of A β 1–42; 3xK also increased the level of A β 1–43. K624E produced shorter A β peptides, primarily A β 1–33 and A β 1–37; although the shift was not as dramatic as that observed with K624A, it is consistent with previous studies.^{20,41} Although no changes were observed with respect to ϵ -cleavage site utilization in the K624A mutant,³³ we nevertheless examined the initial ϵ -cleavage site utilization with the novel mutants that showed the most dramatic changes. Studies for detecting AICD and evaluating ϵ -cleavage site

utilization from the stable CHO lines were unsuccessful. However, using recombinant WT C100Flag, 3xK-C100Flag, and K28E-C100Flag (K28 is equivalent to K624E in the C-100 construct) as substrates in *in vitro* γ -secretase assays, we were able to detect AICD and ϵ -cleavage site utilization. C50–99 and C49–99 were found to be the predominant cleavage products from WT, 3xK, and K28E substrates, and the relative levels of each AICD were unchanged (Figure 1E). Using this assay and recombinant K28E/G29E/A30E (3xE)-C100Flag substrate, we also explored how this mutant would influence A β and AICD profiles (Figure 1 and Table 1 of the Supporting Information). A β 40 and A β 42 were the major products, and the AICD fragments of 3xE-C100Flag were similar to that of WT-C100Flag. Overall, these data confirm our previous observations that reducing the charge at the interface of the ectodomain and TMD increases γ -secretase processivity and increasing the positive charge can in some instances dramatically decrease processivity with a minimal effect on initial ϵ -cleavage site utilization. A single negative charge increases processivity; however, an increased number of negative charges at the interface appears to decrease processivity.

Effects of GSMs and iGSM on the A β Profiles of the Mutants. To determine whether the mutant constructs were responsive to GSMs and iGSMs, GSM-1, a piperidine acetic acid as a potent acidic GSM,⁴² Compound 2 (Cmpd2), a piperazinyl pyrimidine as a potent nonacidic GSM,¹⁹ and fenofibrate, one of the more potent iGSMs identified,²² were utilized. These compounds are depicted in Figure 2A along with EC₅₀ and EC₉₀ values (empirically determined) for altering A β 42 from CHO cells expressing wild-type APP695 (WT). For these studies, we used the compounds at their approximate EC₉₀ values and compared the change in the A β profile following GSM or iGSM treatment to vehicle (DMSO) controls using the described IP–MS methods. Representative spectra from these studies are shown in Figure 2, with the average percent of total peak height for each A β isoform obtained from multiple experiments graphically depicted in Figure 2 of the Supporting Information. As previously shown and also demonstrated in this study, for wild-type APP, GSM-1 treatment decreased the level of A β 42 and increased the level of A β 38.^{42,55} Cmpd2 decreased the levels of both A β 40 and A β 42 and increased the levels of A β 37 and A β 38.¹⁹ Fenofibrate increased the level of A β 42 and decreased the level of A β 38.²² GSM-1 and fenofibrate (iGSM) have more selectivity in terms of the A β product line (preferred product line of A β 48) than, e.g., Cmpd 2 (nonacidic GSM) that affects both A β 48 and A β 49 product lines (Figure 2B). The modulatory effects of the compounds on the mutants are illustrated in Figure 2C–H and Figure 2 of the Supporting Information. A β enzyme-linked immunosorbent assays were performed to calculate the EC₅₀ values of GSM-1 and Cmpd 2 for decreasing the levels of A β 40 and A β 42 on each of the APP mutant constructs (Tables 2 and 3 of the Supporting Information). The EC₅₀ values of both GSMs gradually increased as the number of positive charges increases. In some cases, EC₅₀ values could not be calculated because of the low levels of A β 40 and A β 42 produced (K624A and K624E) or substrates were not modulated by the GSM (G625K and 3xK).

Because of the complexity of statistically assessing the effect of global changes in A β isoforms from each mutant combined with the drug treatments illustrated in Figure 2, we utilized a previously established method termed the modulation index

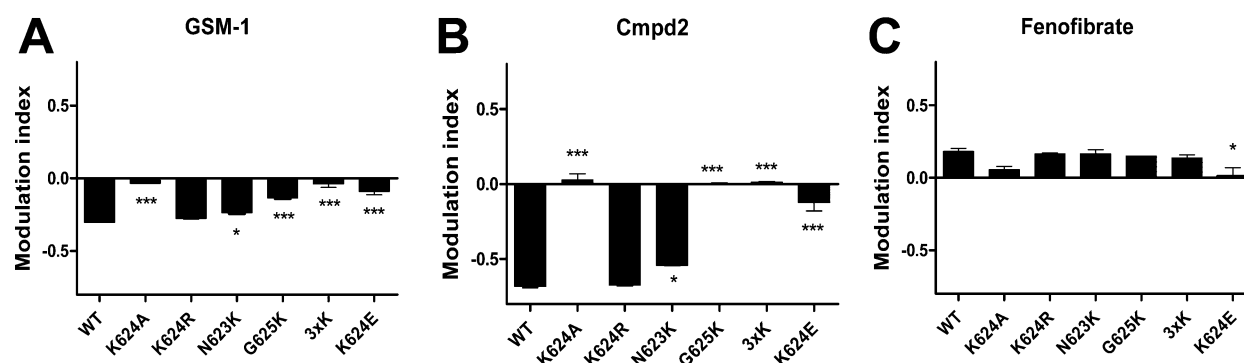


Figure 3. Representation of the substrate responsiveness by GSM and iGSM treatment of the mutants as determined by the modulation index (MI). MI reflects the net changes in each $A\beta$ profile after GSM-1, Cmpd 2, and fenofibrate treatments of the WT and mutant substrates. A negative value indicates GSM activity, while a positive value indicates iGSM activity. (A–C) The WT and K624R did not show any significant change after GSM and iGSM treatment, whereas the mutants with qualitative or quantitative lysine manipulations (K624A, N623K, G625K, 3xK, and K624E) all showed significant decreases in their responsiveness to both classes of GSM relative to the WT. The K624A and 3xK mutants demonstrated a significant reduction in MI. Fenofibrate treatment of the mutants showed no significant difference between the WT and each of the mutants except for K624E. Results were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc testing (* $p < 0.5$, ** $p < 0.01$, and *** $p < 0.001$).

(MI).⁵⁵ The MI can statistically determine whether a treatment modulates the overall $A\beta$ profile. The MI for each mutant and drug combination is shown in Figure 3A–C. These data show that the K624R mutant remains responsive to both GSMs (Figure 3A,B) and the iGSM (Figure 3C) showing relatively unchanged $A\beta$ profiles and MIs relative to those of the WT. In contrast, the other mutations had dramatic effects on compound responsiveness. The K624A mutant showed the most dramatic effect and was no longer responsive to GSMs (Figure 3A,B). The K624A mutant showed a reduced responsiveness to fenofibrate, but the effect was not quite statistically significant ($p = 0.057$) (iGSM) (Figure 3C). Compared to the WT, the N623K mutant showed a weakened response to the acidic GSM-1 (Figure 3A) and the nonacidic Cmpd2 (Figure 3B), with no notable change to the fenofibrate treatment (Figure 3C). The G625K mutant shows a dramatically weakened response to GSM-1, no response to Cmpd2, and a preserved response to fenofibrate (Figure 3). The 3xK mutant showed essentially no response to both GSMs but a preserved response to the iGSM (Figure 3). The K624E mutant showed significantly weakened responses to both GSMs and iGSM (Figure 3).

DISCUSSION

Although we originally hypothesized that the activity of acidic but not nonacidic GSMs would be influenced by single and double amino acid substitutions altering positively or negatively charged residues at the interface of the APP ectodomain and TMD, we find that mutations in this region can dramatically alter responses to both classes of GSMs. In contrast, inhibition of processivity by an iGSM was not significantly altered by mutations other than K624A and K624E. Along with a previous study from our group showing that first-generation NSAID-based GSMs showed a high degree of substrate selectivity for APP relative to modulation of Notch-1 or CD44 cleavage,⁴⁰ these data suggest that the effect of a GSM or an iGSM requires a permissive substrate. We do note that second-generation GSMs have been shown to potentially modulate Notch,^{60,61} however, that effect seems to be much less pronounced than that shown for APP. Here we extend these studies by showing that GSM modulation is highly dependent upon the amino acid sequence near the interface of the ectodomain and TMD.

Notably, as we were preparing these data for publication, Ousson et al. reported similar effects of the K624E mutation on GSM activity.⁴¹ In their study using a signal peptide CTF β construct (SP-C99) containing the K28E mutation, they found a diminished responsiveness to GSM-1 for decreasing the level of $A\beta_{42}$ and a shift in activity for a nonacidic heteroaryl-type GSM (E2012) to an iGSM in the presence of the mutation.

On the basis of the stepwise cleavage model initially proposed by Ihara and colleagues³⁴ along with the data generated in this work and others,^{20,33,40,41} we can propose models depicting how GSMs, iGSMs, and various mutations that alter charge at the interface of the APP ectodomain and TMD modify γ -secretase processivity (Figure 4). Although for the sake of simplicity we illustrate increased processivity producing shorter forms of $A\beta$ with an increased number of tri- or tetrapeptide cleavages, it is possible that increased processivity could also occur through larger stepwise cleavages. These models assume that the active site of γ -secretase is relatively fixed in position and that following the initial ϵ -site cleavage the substrate “sinks” into or is “pulled down” into the active site, resulting in the stepwise cleavages. Such a model is consistent with the proposed structural models of γ -secretase that are based on the crystal structure of signal peptide peptidases.⁶² Although it is formally possible that the active site of γ -secretase moves relative to the substrate, this would seem to be highly thermodynamically unfavorable for a structurally constrained membrane-embedded protease.⁶³ In contrast, it is well-established that small transmembrane peptides can be mobile, both laterally and vertically within the membrane.⁶⁴ For the cleavage of wild-type APP, typically three or four successive cleavages by γ -secretase occur along the two major product lines, resulting in the normal profile of $A\beta$ species (Figure 4A). In the K624A mutant, loss of the positively charged lysine residue appears to permit $A\beta$ to sink further into the active site. One or two additional tri- and tetrapeptide cleavages would then generate the major species seen with this construct, $A\beta_{33}$ and $A\beta_{34}$ (Figure 4B). Because our study is based on cell-based assays, we did not attempt to detect the small peptides sequentially released to produce the shorter $A\beta$ peptides. However, considering that there were no alterations in the major AICD fragments,³³ the shorter $A\beta$ isoforms (i.e., $A\beta_{33}$ or

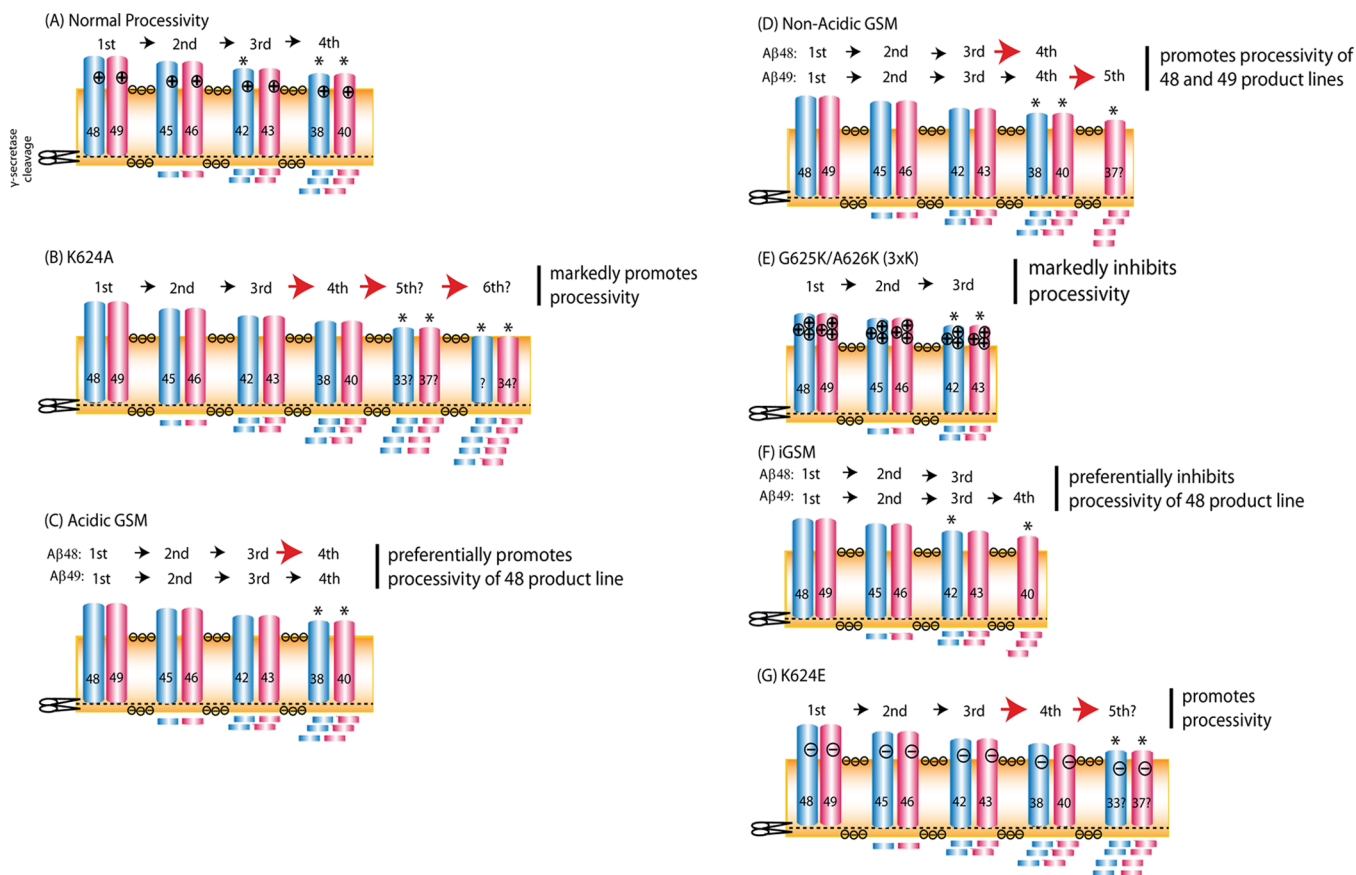


Figure 4. Proposed model of γ -secretase processivity based on charge manipulation or treatment with a GSM or an iGSM. (A) γ -Secretase cleavage occurs near the cytoplasmic face of the TMD. The major $A\beta$ product lines, $A\beta$ 48 and $A\beta$ 49, are colored blue and red, respectively. The plus signs indicate positive charge at the JMD–TMD region, representing the membrane-anchoring residue at the luminal side. Each cytosolic fragment represents sequential tri- or tetrapeptide cleavage products. $A\beta$ isoforms secreted and detected from the conditioned media are denoted with asterisks. Typically, both the $A\beta$ 48 and $A\beta$ 49 products undergo up to four rounds of cleavage in the processivity model. (B) Neutralization of the charge leads to the release of shorter $A\beta$ isoforms such as $A\beta$ 33, $A\beta$ 34, and $A\beta$ 37, which would be generated after the fifth or sixth cleavage. This mutation appears to significantly promote processivity. (C) Acidic GSMs have selectivity for modulation of the $A\beta$ 48 over the $A\beta$ 49 product line by promoting its third cycle, resulting in an increase in the level of $A\beta$ 38 formation with minimal effects on $A\beta$ 40 levels. (D) Nonacidic-type GSMs promote processivity of both the $A\beta$ 48 and $A\beta$ 49 product lines, with the third cleavage of $A\beta$ 48 and the fourth cleavage of $A\beta$ 49 increasing, respectively. (E) Increased charge is hypothesized to hold the substrate tight at the luminal site because of the interaction between the positively charged amino acids and the phosphate group of the phospholipids, thus preventing the substrate from being pulled down to the cytosolic side. This results in an increased level of release of longer $A\beta$ isoforms, i.e., $A\beta$ 42 or $A\beta$ 43. (F) Similar to the acidic GSMs, the iGSM showed selectivity for the $A\beta$ 48 product line. It is suggested that by an as yet unknown mechanism, an iGSM perhaps inhibits the movement of the substrate toward the γ -sites. (G) The negative charge replacement at the interface of the transmembrane protein appears to increase processivity. In contrast to the positively charged mutants, the negatively charged amino acid at the JMD–TMD region would be predicted not to interact with the phospholipids, thus allowing more vertical flexibility in the lipid bilayer for the substrate to further dip into the active site.

$A\beta$ 34) are almost certainly derived by enhanced stepwise processivity.

Both acidic and nonacidic GSMs also promote processivity but in distinct fashions. Acidic GSMs primarily promote single-step processivity on the $A\beta$ 48 to $A\beta$ 42 product line, resulting in a decreased level of $A\beta$ 42 and an increased level of $A\beta$ 38 (Figure 4C). In addition to its primary effect, acidic GSM (GSM-1) not only affects the cleavage from $A\beta$ 42 to $A\beta$ 38 but also affects the shift from either $A\beta$ 41 or $A\beta$ 43 to $A\beta$ 38 by releasing a different number of amino acids ranging from three to five.³⁹ Nonacidic GSMs promote an additional cleavage along both product lines, resulting in less $A\beta$ 40 and $A\beta$ 42 and more $A\beta$ 38 and $A\beta$ 37 (Figure 4D).

In contrast to these alterations that promote processivity, increasing charge decreases processivity along one product line (e.g., G625K) or both (3xK) (Figure 4E). In this case, rather than three or four cleavage steps, these mutant APPs are more

typically processed only two or three times. The positive charge is predicted to interact with the negatively charged phosphate group of the phospholipids in the lipid bilayer, and this interaction perhaps prevents the transmembrane protein from sinking down to the ϵ -cleavage site. Finally, iGSMs appear to preferentially reduce processivity by one cycle along the $A\beta$ 42 product line (Figure 4G). As long as the positive charge (K624R, N623K, G625K, and 3xK) is preserved, the iGSM is capable of increasing the level of $A\beta$ 42, yet when the positive charge was replaced with a noncharged or negatively charged amino acid, iGSM activity was markedly reduced. Given our observations with regard to iGSM activity, the mechanism of action for an iGSM is hypothesized to be similar to that of the acidic-type GSM but in the opposite manner.^{22,65}

Our study of the effects of negatively charged amino acid substitutions at the JMD–TMD region on $A\beta$ production further validates previous findings.^{20,41} The K624E mutant

generates more of the shorter $A\beta$ species such as $A\beta_{33}$ and $A\beta_{37}$. As explained above for the 3xK mutant, because the phosphate group has a negative charge, the K624E residue may sit with the phosphate group driving the $A\beta$ production more like the noncharged mutant allowing the construct to sink a bit further down than the WT (single lysine). Although the K624E mutant allows more flexibility in the vertical movement of the transmembrane protein, the presence of charge may still prevent the protein from moving down deeper to the hydrophobic region of the phospholipids. This could also explain why our 3xE mutant showed decreased processivity compared to that of the K624E mutant (Figure 4G and Figure 1 of the Supporting Information).

In this model, the effects of decreasing or increasing the charge at the interface of the APP ectodomain and TMD on γ -processivity fit well with the concept that positive charge in this portion of APP could regulate the extent to which the substrate moves relative to the active site of γ -secretase. In contrast, the effects of GSMs are more challenging to explain. On the basis of the observations that (i) the substrate sequence dramatically alters the action of a GSM and (ii) photoaffinity labeling⁴⁷ and nuclear magnetic resonance (NMR) data⁴⁶ show that NSAID-based acidic GSMs can bind the GXXXG motif carboxyl to K624A in APP, and (iii) affinity and labeling studies show that GSM-1 binds PSEN1^{66,67} and nonacidic GSMs targeting either PSEN1 or PEN-2,^{68–70} we suggest that there is complex interaction among γ -secretase, its substrate, and various GSMs. In some cases, there might be a tripartite interaction among the substrate, GSM, and γ -secretase that directly influences processivity by influencing the extent to which the substrate can “dip” into the active site following each successive cleavage. In other cases, the GSM may have an allosteric effect on the γ -secretase complex that indirectly influences the interaction of the substrate with the enzyme. It is also possible that some GSMs work through a combination of allosteric effects on γ -secretase and tripartite interactions with the enzyme and substrate. The membrane lipid may also regulate processivity and GSM action.⁷¹ In any of these scenarios, single-point mutations can completely block the ability of a GSM to modulate processivity or as reported by others actually switch the activity of a GSM to that of an iGSM.⁴¹ Such data suggest an interdependence of the modulatory effect of these compounds on substrate sequences, and further reinforce the novelty of this mechanism of altering proteolytic cleavage.

In conclusion, we find that effectiveness of both major classes of GSMs identified to date is highly influenced by the presence of positively charged residues at the interface of the APP ectodomain and TMD. Replacement of the positive charge in the K624A or K624E construct or increasing the positive charge in the G625K and 3xK construct either completely blocks or attenuates the effectiveness of these GSMs. Furthermore, both GSMs and mutations that alter the processive cleavage by γ -secretase do not appear to significantly alter ϵ -cleavage. This cleavage and not the subsequent stepwise processivity is thought to be the key determinant for potential toxicities related to the inhibition of γ -secretase. Thus, the collective data suggesting substrate specificity and the lack of effect on ϵ -cleavage reinforce the potential intrinsic safety of GSMs as prophylactic therapeutics for the treatment of AD.^{16,20,33,37,40,41,72,73}

■ ASSOCIATED CONTENT

📄 Supporting Information

Supplementary figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ABBREVIATIONS

$A\beta$, amyloid β ; AD, Alzheimer's disease; AICD, amyloid precursor protein intracellular domain; APP, amyloid precursor protein; CHO, Chinese hamster ovary; Cmpd2, Compound 2; CTF β , carboxyl-terminal fragment β ; DMSO, dimethyl sulfoxide; GSI, γ -secretase inhibitor; GSM, γ -secretase modulator; iGSM, inverse γ -secretase modulator; IP-MS, immunoprecipitation and mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MI, modulation index; NSAID, nonsteroidal anti-inflammatory drug; PSEN1, presenilin 1; PEN2, presenilin enhancer 2; SEM, standard error of the mean; EC₅₀, half-maximal effective concentration; WT, wild type.

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