Aberrant Amyloid Precursor Protein (APP) Processing in Hereditary Forms of Alzheimer Disease Caused by APP Familial Alzheimer Disease Mutations Can Be Rescued by Mutations in the APP GxxxG Motif^{*}

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The identification of hereditary familial Alzheimer disease (FAD) mutations in the amyloid precursor protein (APP) and presenilin-1 (PS1) corroborated the causative role of amyloid- β peptides with 42 amino acid residues (A β 42) in the pathogenesis of AD. Although most FAD mutations are known to increase Aβ42 levels, mutations within the APP GxxxG motif are known to lower A β 42 levels by attenuating transmembrane sequence dimerization. Here, we show that aberrant A β 42 levels of FAD mutations can be rescued by GxxxG mutations. The combination of the APP-GxxxG mutation G33A with APP-FAD mutations yielded a constant 60% decrease of AB42 levels and a concomitant 3-fold increase of A β 38 levels compared with the Gly³³ wild-type as determined by ELISA. In the presence of PS1-FAD mutations, the effects of G33A were attenuated, apparently attributable to a different mechanism of PS1-FAD mutants compared with APP-FAD mutants. Our results contribute to a general understanding of the mechanism how APP is processed by the γ -secretase module and strongly emphasize the potential of the GxxxG motif in the prevention of sporadic AD as well as FAD.

 APP^2 and APLPs were conventionally thought to exist and to act as monomers. However, biochemical and structural data have accumulated over the past few years, indicating that APP and APLPs exist as functional dimers or even are present in higher oligomeric units (1–6). Interactions of APP and APLPs



APP is first cleaved by the β -site APP cleaving enzyme and is then sequentially processed by the γ -secretase complex to generate A β peptides of varying length (10, 11). γ -Secretase cleavage specificity is modulated by the *GxxxG* ("G-triple-*x*-G") dimerization motif of the APP-TMS, and we showed previously that APP can be cleaved as a homodimer by β - and γ -secretases (9). APP, APLP1, and APLP2 share similar interaction motifs and can form APP-APLP1 and APP-APLP2 complexes (7). Cotransfections of APP with APLP1 or APLP2 influenced APP processing into A β leading to decreased A β 40 and A β 42 levels likely through an influence on γ -secretase cleavages (7).

According to the amyloid hypothesis, $A\beta$ peptides represent the main culprit of Alzheimer disease (AD). Based on this assumption is the appealing prediction that reducing A β levels would ameliorate Alzheimer symptoms (12, 13). In the current model of A β generation, the initial cut at the ϵ -site is executed by the presenilins of the γ -secretase complex, leading to formation of the APP intracellular domain and AB49 or AB48 peptides (10, 14). The latter two likely remain bound to the active site and are successively cleaved every three to four residues at the ζ -site and at the γ -sites (11, 15). Most likely, two product lines exist. In the product line encompassing A β 40, A β 49 is trimmed to A β 40, and in the other product line, A β 48 is the precursor of A β 42 and A β 38 (10, 15). When we studied homodimerization of APP-TMS, a bacterial test system revealed that glycine residues 29 and 33 of the GxxxG amino acid motif represent the major helix-helix contact site (see Fig. 1A). Substitutions of either of the two glycine residues not only diminished APP-TMS homodimer stability but also drastically decreased AB42 and increased AB38 levels, among other changes (9). Our findings that the γ -secretase complex can cleave dimeric substrates complement the current model of successive cleavages producing A β peptides from two predominant product lines (15).

Importantly, mutations in three different genes are associated with AD and were described to increase the ratio of A β 42 to A β 40 in mice and humans (16–18). Early onset dominant

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² The abbreviations used are: APP, amyloid precursor protein; sw, Swedish; Aβ, amyloid-β peptide; AD, Alzheimer disease; ELISA, enzyme-linked immunosorbent assay; FAD, familial Alzheimer disease; GSM, γ-secretase modulators; sAPPα/-β, secreted APP ectodomain α- or β-cleaved; sw, Swedish FAD mutation; TMS, transmembrane sequence; PS, presenilin; wt, wild-type; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; ANOVA, analysis of variance; APLP, APP-like protein.

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A β Generation Is Determined by the APP GxxxG Motif

FIGURE 1. **APP- and PS1-FAD mutations.** *A*, part of the APP sequence is shown. Indicated are the β - and α -secretase cleavage sites as well as all analyzed APP-FAD mutations. The glycine residues mediating the APP-TMS dimerization (9) are highlighted (Gly²⁹/Gly⁷⁰⁰ and Gly³³/Gly⁷⁰⁴ according to A β or APP770 *numbering*, respectively). *B*, APP-FAD and APP-FAD-G33A constructs are equally well and stably expressed in the SH-SY5Y cells. The *double band* represents mature, plasma membrane-residing (~130 kDa) and immature (~110 kDa) APP, and Western blot was stained with 22C11. As loading control, actin was stained as shown in the *lower panel* (~40 kDa). *C*, an amino acid sequence of PS1 TMS-7 is shown. Analyzed FAD mutations and the catalytically active Asp³⁸⁵ are highlighted. The *gray boxes* in *C* mark residues embedded in the cell membrane.

AD was first associated with the APP gene and found to alter the amount of $A\beta$ produced and/or the ratio of $A\beta$ 42 to $A\beta$ 40 (16, 19). Subsequently, early onset familial mutations in the presenilins were also linked to AD (17, 20). Using some of these mutations, we further investigated the *Gxxx*G-mediated effects on γ -secretase cleavage by combining *Gxxx*G mutations with FAD mutations.

We found here a constant 60% decrease in A β 42 and a 3-fold increase in A β 38 levels for APP-FAD mutants when we impaired the APP-GxxxG-mediated interaction by introducing the mutation G33A. However, G33A only had mild effects in the presence of PS1-FAD mutations. Furthermore, we found that the analyzed FAD mutants can be divided into subgroups: (i) APP-FAD mutants that increase A β 42 and A β 38 levels and either show a concomitant increase or a decrease of A β 40 levels, (ii) APP-FAD mutants that do not affect A β 42 and A β 38 levels but exclusively decrease A β 40 levels, and (iii) PS1-FAD mutants that increase A β 42, but decrease A β 38 levels.

EXPERIMENTAL PROCEDURES

Plasmids and Transfections—The plasmid pCEP4 containing the coding sequence for APP695 with an N-terminal Myc tag and C-terminal FLAG tag was used as a template to introduce the APP-FAD mutations and G33A by site-directed mutagenesis. Either G33A or the FAD variants were used to generate the double mutant constructs APP-FAD-G33A in a second sitedirected mutagenesis. The same method was applied to generate SPA4CT-G33A from pCEP4-SPA4CT with a C-terminal FLAG tag and the PS1-FAD mutants from pcDNA3.1/zeo (Invitrogen) containing the PS1coding sequence with an N-terminal hemagglutinin tag. SPA4/1-52 and SPA4/1-51 were generated from pCEP4-SPA4CT by replacing codons 53 or 52 by stop codons, respectively. All sequences were confirmed by dideoxy sequencing and restriction digestion. For stable expression of APP or SPA4CT constructs, plasmids $(3 \mu g)$ were transfected into SH-SY5Y cells (ATCC catalog no. CRL-2266) at 9 \times 10⁵ cells/6-well or 80% confluency using Transfectine (Bio-Rad) or Lipofectamine and Plus reagent (Invitrogen) following the manufacturer's instructions. Stably transfected cells were selected with hygromycin (250 µg/ml). SPA4CT-expressing cell lines were co-transfected with the PS1 constructs using Lipofectamine and Plus reagent and were additionally selected with zeocin (200 μ g/ml). Two to three independent stable cell lines were generated.

Cell Culture, Sandwich ELISA, Immunoprecipitation, Western Blot,

and MALDI-MS-SH-SY5Y cells routinely were cultured as described (9). $A\beta 40/A\beta 42$ ELISAs were performed according to the manufacturer's instructions (The Genetics Company). The following antibody combinations were used: for A β 38, BA1–13 (Covance/Signet) and W0-2-biotin (TGC); for sAPP α , anti-Myc antibody (Cell Signaling Technology) and W0-2-biotin; and for total A β , W0-2 or 4G8 (Covance) and 4G8-horseradish peroxidase (Covance) or W0-2-biotin, respectively. W0-2-biotin was recognized by streptavidin-conjugated horseradish peroxidase, and ELISAs were developed with 1-Step Ultra-TMB (Pierce) and were measured at 450 nm in a microplate reader (Anthos). Western blot analyses and immunoprecipitations were performed as published (9). Briefly, 250 μ l of conditioned media were diluted with phosphate-buffered saline and precipitated with polyclonal rabbit antibody 18-1 (generated to A β 1-40) or W0-2 and protein A- or G-Sepharose (GE Healthcare), respectively. For Western blot analysis, cells were lysed in a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 1% Nonidet P-40. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and immunostained. For MALDI-MS analyses, A β was eluted twice from Sepharose with 500 µl 50% acetic acid, and the eluate was vacuum-dried. MALDI-MS analysis was carried out as described previously (9).

Structural Model—The APP-TMS model from previous analyses based on the tertiary structure of glycophorin A was used as a template (9). The FAD mutants were added to the structure using the Swiss-PdbViewer. The most likely rotamers were selected before the model was energetically minimized





FIGURE 2. **Analysis of APP-FAD mutations.** Quantification of secreted sAPP α , total A β , A β 42, A β 40, and A β 38. A, sAPP α levels, APP-wt (without FAD) set as 100% (mean \pm S.E., n = 5-16). B, total secreted A β levels, APP-wt set as 100% (mean \pm S.E., n = 6-14). Note that A692G and E693G could not be analyzed by total A β ELISA as the FAD mutations alter the epitope of the antibody 4G8. C, total A β levels of A692G and E693G were analyzed by immunoprecipitation with antibody 18-1 and Western blot analysis with W0-2. D, A β 42 levels, APP-wt set as 100% (mean \pm S.E., n = 5-17). E, A β 38 levels, APP-G33A set as 100%, see supplemental Fig. S1C and (mean \pm S.E., n = 4-12). F, A β 40 levels, APP-wt set as 100% (mean \pm S.E., n = 7-16). A-E, asterisks indicate significant differences to APP-wt (*, p < 0.01, **, p < 0.001, one-way ANOVA type Dunnett). Shaded bars indicate mutations increasing A β 42 and A β 38. Horizontal shaded bars highlight mutations only decreasing A β 40. Horizontal lines mark the fragment levels of APP-wt for better comparison with the mutants.

applying the GROMACS Software. All figures were produced with the PyMOL Molecular Graphics System.

RESULTS

Analysis of APP-FAD Mutants-We analyzed APP processing of nine APP-FAD mutations scattered around the three secretase cleavage sites, *i.e.* K670N/M671L at the β-site (Swedish, hereafter referred to as "sw"), A692G (Flemish) and E693G (Arctic) near the α -site, and T714I (Austrian), V715M (French), I716V (Florida), V717G, V717F, and L723P (Australian) at the γ -secretase cleavage sites (Fig. 1A) (19, 21–28). The APP-FAD mutants were stably expressed in SH-SY5Y cells and yielded similar expression levels as determined by Western blot analysis (Fig. 1*B*). The levels of sAPP α , A β 42, A β 40, and A β 38 were quantified by sandwich ELISA (Fig. 2). For the APP-sw mutation, sAPP α levels were found to be reduced by 80%, and total A β levels were increased, whereas all other APP-FAD mutants showed total A β levels comparable to APP-wt (Fig. 2, A-C). In five out of nine variants (sw, A692G, T714I, V715M, and V717F) increased AB42 levels were observed compared with APP-wt (Fig. 2D), whereas the other four FAD variants, i.e.

E693G, I716V, V717G, and L723P only reached AB42 levels of APP-wt. Thus, increased A β 42 levels are not a general feature of APP-FAD mutations. For six out of nine FAD variants, AB38 levels were increased compared with APP-wt (Fig. 2E). Interestingly, all five mutations that resulted in increased AB42 levels also showed increased Aß38 levels (Fig. 2, D and E, shaded bars). AB40 levels were found elevated only for those FAD mutations that reside within the N-terminal half of the $A\beta$ sequence. In contrast, a significant decrease of $A\beta 40$ was observed for all six FAD mutations located within the C-terminal region of the TMS (Fig. 2F). FAD mutants that do not affect A β 38 or A β 42 levels, but solely decrease A β 40 levels are I716V, V717G, and L723P (Fig. 2F, horizontal shaded bars). The Arctic APP mutation E693G is an exception insofar as it only increased A β 38 levels. Interestingly, the APP-sw mutant yielded 4-fold enhanced AB42 and AB38 levels, but only 2-fold enhanced A β 40 levels (9, 15). Thus, the sw mutation might not exclusively affect the β -secretase cut as predicted (19). We clearly observed that amino acid exchanges in the AB N-terminal region (sw, Flemish, and Arctic) can influence γ -secretase processing.





Effect of the GxxxG Motif on APP-FAD Processing—To investigate the role of the GxxxG motif known to mediate APP-TMS dimerization, we tested constructs encoding individual APP-FAD mutations in combination with the GxxxG mutation G33A (APP-FAD-G33A). All APP-FAD-G33A-derived constructs generated sAPP α and total A β levels similar to the respective APP-FAD variant alone, indicating that their processing is equally efficient as that of APP-wt or APP-FAD alone (supplemental Fig. S1). However, the striking difference is that the APP-FAD-G33A constructs drastically reduced A β 42 levels compared with APP-FAD alone (Fig. 3*A*). Mutants A692G/G33A, T714I/G33A, and V715M/G33A yielded A β 42 levels similar to APP-wt. The mutants E693G, I716V, V717G, V717F, and L723P in combination with G33A did not even reach A β 42 levels of APP-wt. However, the APP-sw-G33A mutant still produced 1.6-fold higher A β 42 levels than APP-wt (Fig. 3*A*). For better comparison and to calculate the percent impact of G33A on processing, data of Gly³³-wt were each normalized to 100% (Fig. 3, *B–D, gray bar*) and are displayed for comparison to G33A (Fig. 3, *B–D, black bars*). For all nine APP-FAD muta-



tions tested, the G33A mutation yielded a reduction of A β 42 levels by ~60% (Fig. 3*C*) and a 3-fold increase of A β 38 levels (Fig. 3*D*), whereas A β 40 levels remained unchanged (Fig. 3*B*). Thus, the G33A mutation within the G*xxx*G motif possesses the same strong impact on APP-FAD mutations as on APP-wt in terms of decreasing A β 42 production and thus has a FAD-rescuing effect. By MALDI-MS analyses, we confirmed that no construct generated an aberrant pattern of A β species (supplemental Fig. S2). This data indicates that single amino acid changes within the G*xxx*G motif can rescue APP-FAD-related effects.

This observation raised the question of whether G33A affects the processing at early (ϵ -site) or subsequent (γ -sites) cleavage steps of γ -secretase. Therefore, two extended constructs were generated encoding elongated A β species that were shown to be either processed as precursors of the A β 42 line, *i.e.* A β (1– 51), or by the A β 40 line, *i.e.* A β (1–52) (29). As these constructs were generated from the C-terminal 99 residues of APP (SPA4CT) (30), the fragments are named SPA4/1-51 and SPA4/1-52. The constructs are normally expressed in SH-SY5Y cells and processed like the C-terminal fragment of APP (Fig. 3*E*). The quantification of A β levels shows that SPA4/ 1–52 predominantly generates A β 40 but neither A β 42 nor A β 38 (Fig. 3*F*). SPA4/1–51 generates mainly A β 42 and A β 38. SPA4/1-51 containing the mutation G33A shows 2.5-fold reduced AB42 levels and 2-fold increased AB38 levels compared with Gly³³-wt. Aβ40 levels are not affected in both constructs. Thus, the mutation G33A similarly influences processing of SPA4/1-51, SPA4/1-52, APP-wt, and APP-FAD. This indicates that the mutation G33A has a universal effect on A β 42 and A β 38 generation. Importantly, the result also shows that the allocation to one or the other product line cannot be modulated by G33A mutation.

Analysis of PS1-FAD Mutants—Three mutations, i.e. G378A, L381A, and G384A, located near or within the highly conserved GXGD motif of PS1 in close proximity to the catalytically critical aspartate residue 385 within TMS-7 (31–33) were analyzed (Fig. 1C). As substrate, we used SPA4CT instead of the fulllength APP, as SPA4CT yields higher A β levels (9). Stably expressing SH-SY5Y cell lines were selected for expression of similar levels of holo-PS1, the N-terminal fragment of PS1, and SPA4CT by Western blot analysis (supplemental Fig. S3). Efficiency of SPA4CT processing by PS1-FAD mutants did not vary between cell lines as indicated by total A β levels measured (Fig. 4A). All three PS1-FAD mutants led to increased levels of AB42 and decreased levels of A β 38 (Fig. 4, C and D). A β 40 levels were found specifically reduced for PS1-G384A (Fig. 4B), a mutant that has already been described to selectively lower AB40 production (34). Thus, an obvious difference between the investigated PS1-FAD and APP-FAD mutations are the consistently increased A β 42 levels of PS1-FAD at the expense of decreased Aβ38 levels, whereas APP-FAD mutants that increase Aβ42 also increase A β 38 levels. This also indicates that PS1-FAD processing is divergent from APP-FAD.

Impact of the GxxxG Mutation G33A on PS1-FAD Processing—Effects of the GxxxG motif on APP and SPA4CT processing are very similar, as described previously (9). Now, to investigate possible GxxxG-mediated influences on PS1-



FIGURE 4. **Analysis of PS1-FAD mutants in the Gly³³-wt background.** Quantification of secreted A β from SH-SY5Y cells coexpressing PS1-FAD mutants and SPA4CT-wt. PS1-wt was set as 100%. *A*, total A β levels (mean \pm S.E., n = 3-10). *B*, A β 40 levels (mean \pm S.E., n = 3-10). *C*, A β 42 levels (mean \pm S.E., n = 3-8). *D*, A β 38 levels (mean \pm S.E., n = 3-9). Asterisks indicate significant differences to PS1-wt/SPA4CT-wt (*, p < 0.01, **, p < 0.001, one-way ANOVA type Dunnett).



FIGURE 5. **Impact of the GxxxG mutation G33A on PS1-FAD processing.** ELISA data from PS1-FAD and SPA4CT-G33A coexpressing cells (*black bars*) in comparison to the respective SPA4CT-wt expressing lines, set as 100% (*gray bars*). *A*, total A β levels (mean \pm S.E., n = 3-10). *B*, A β 40 levels (mean \pm S.E., n = 3-10). *C*, A β 42 levels (mean \pm S.E., n = 3-8). *D*, A β 38 levels (mean \pm S.E., n = 3-9). *A*, Ab40 levels (mean \pm S.E., n = 3-9). *A*, Ab40 levels (mean \pm S.E., n = 3-9). *A*, Ab40 levels (mean \pm S.E., n = 3-9). *A*, Ab40 levels (mean \pm S.E., n = 3-9). *A*, Ab40 levels (mean \pm S.E., n = 3-9). *A*, Ab40 levels (mean \pm S.E., n = 3-8). *D*, Ab40 levels (mean \pm S.E., n = 3-9). *A*, Ab40 levels (mean \pm S.E., n = 3-9). *A*, Ab40 levels (mean \pm S.E., n = 3-9). *A*, Ab40 levels (mean \pm S.E., n = 3-9). *A*, Ab40 levels (mean \pm S.E., n = 3-9). *A*, Ab40 levels (mean \pm S.E., n = 3-9). *A*, Ab40 levels (mean \pm S.E., n = 3-9). *A*, Ab40 levels (mean \pm S.E., n = 3-9). *A*, Ab40 levels (mean \pm S.E., n = 3-9). *B*, Ab40 levels (mean \pm S.E., n = 3-9).

FAD mutants, we stably coexpressed SPA4CT-G33A and PS1-FAD mutants in SH-SY5Y cells and determined levels of $A\beta$ in the cell culture supernatants by ELISA (Fig. 5 and supplemental Fig. S4). $A\beta$ levels generated from Gly³³-wt substrates were set as 100%. Total $A\beta$ levels were similar for all constructs tested indicating that the SPA4CT-G33A protein was efficiently processed by the respective PS1-FAD γ -secretase complex (Fig. 5*A*). As expected for the G33A mutant, $A\beta$ 40



FIGURE 6. **Model of the pathological mechanisms caused by APP-FAD and PS1-FAD mutations.** Oxygen atoms are depicted in *red*, nitrogen atoms are depicted in *blue*, and sulfur atoms are colored *dark yellow*. The glycine residues of the interface are highlighted in *yellow*, and APP-FAD mutations are colored *pink*. *Scissors* indicate peptide bonds that are cleaved by the γ -secretase. *A*, dimeric wt substrates are degraded by the γ -secretase predominantly by the A β 40 line. *Left*, corresponding peptide bonds are indicated and are located at the dimer interface. We propose that APP-FAD mutations cause a general shift between the two product lines so that the A β 40 line is down-regulated, and the A β 42 line becomes a major degradation pathway. *Right*, APP-FAD, peptide bonds of the A β 42 line are indicated. Note, that amino acid side chains from FAD mutations do not reach into the dimer interface. PS1-FAD mutants seem to cause substrate flux inhibition leading to a retarded processing within the A β 42 line causing increased A β 42 and decreased A β 38 levels (*vertical arrow*). The dimer crossing point mediated by Gly²⁹ (Gly⁷⁰⁰) and Gly³³ (Gly⁷⁰⁴) may cause a steric hindrance and inhibit the consecutive γ -secretase processing leading predominantly to A β 40 (β 40 (β 40 line) or to A β 42 ($A\beta$ 42 line) (9, 10). Mechanistically, the effect of G33A occurs after the effects of APP-FAD mutations explaining why G33A causes a constant reduction by 60% for A β 42 and a 3-fold increase of A β 38 in the presence of all individual APP-FAD mutations analyzed. *B*, the APP-FAD-TMS dimer in *side view* for better illustration of the FAD-causing amino acid side chains. T714I, V715M, I716V, and V717F stick out of the dimer interface and thus likely affect processing by modulating the substrate-enzyme recognition.

levels were not affected (Fig. 5*B*). However, the G33A mutation showed the tendency to decrease A β 42 levels (Fig. 5*C*) and to increase A β 38 levels; the latter at least holds true for the G387V and L381V mutations compared with Gly³³-wt (Fig. 5*D*). Attributable to the close proximity to the active site the G384A mutation might follow a different mode of action. There were no aberrant patterns of A β species (supplemental Fig. S5). Although the effect of G33A on A β 42 and A β 38 levels in the presence of PS1-FAD mutations was visible, it was not as strong as observed for the APP-FAD mutations.

DISCUSSION

Processing of APP-FAD and PS1-FAD Mutants—Here, we describe a previously unrecognized relationship between mutations in juxtamembrane positions and γ -secretase cleavages, although the mutations are located $\sim 20-40$ amino acid residues apart from the γ -cleavage sites. This is exemplified by the mixed effect found on A β species for the APP-sw mutant showing a 4-fold increase in A β 42 and A β 38 levels but an only 2-fold increase A β 40 levels. So far, influences on A β 42/A β 40 ratios by APP-sw were only described to vary with the age of mice expressing APP-sw (35) or APP-sw and V717F (36).

We also observed that most of the APP-FAD mutants either increase A β 42 and A β 38 levels or solely decrease A β 40 levels. We explain this observation with the sequential cleavage model of γ -secretase proposing the existence of two product lines (9, 15), the A β 40 line and the A β 42 line (Fig. 6A). We suggest that APP-FAD mutations cause a general shift away from the A β 40 line toward the A β 42 line, explaining why A β 40 levels were found decreased and both A β 42 as well as A β 38 levels were increased (Fig. 6A). Interestingly, molecular modeling reveals that the amino acid side chains of APP-FAD mutations stick out from the APP-TMS dimer interface and thus unlikely impair APP-TMS dimerization in a cellular environment (Fig. 6, A and B). However, Gorman et al. (37) observed an impact of FAD mutations on oligomerization of synthetic peptides in micelles. From our data, we speculate that APP-FAD mutations, especially those located in the C-terminal region, may shift the affiliation to the A β 42 product line by affecting enzyme-substrate recognition rather than that they impair TMS dimerization itself. Thus, the initial recognition of APP-FAD mutations seems to decide on the pathway followed (A β 42 or A β 40 line). This view is supported by results describing that APP-FAD mutations in the C-terminal region affect ϵ -cleavage, leading to an increase of APP intracellular domain levels (APP intracellular domain) promoting the A β 42 line (38, 39). Although the α -helices likely need to be unfolded prior to γ -secrease cleavages, it might be of interest that the peptide bonds cleaved in the A β 42 line reside outside the dimer, whereas the peptide bonds of the A β 40 line reside within the dimer interface.

For the PS1-FAD mutants analyzed, a reduction in A β 38 but an increase in A β 42 levels was the major change observed, indicating that PS1-FAD mutations generally act differently from



APP-FAD mutations. In agreement with the sequential cleavage model, PS1-FAD mutations might lead to an inhibition of flux through the A β 42 pathway, which could account for the decrease of A β 38 and increase of A β 42 levels (Fig. 6*A*). The inhibition of cleavage flux is in agreement with the loss-of-function hypothesis of PS1-FAD mutations (40). The apparently different APP-FAD and PS1-FAD mechanisms question cell culture or mouse models where these mutations are combined to accelerate the A β production and pathology.

Impact of the GxxxG Motif—We have reported previously that the sequence motif GxxxG within the APP-TMS has a regulatory impact on the $A\beta$ species produced. The mutation G33A attenuated the TMS dimerization strength by 20%, specifically reduced the formation of A β 42 by 60%, left A β 40 levels unaffected, but increased the formation of A β 38 (3-fold) and shorter A β species (9). When we analyzed the GxxxG motif in combination with APP-FAD mutations, we found that the G33A mutant yielded the same shift, *i.e.* a 60% decrease of A β 42 levels and a concomitant 3-fold increase of AB38 levels for all APP-FAD mutants. Thus, G33A in combination with APP-FAD mutations affected γ -secretase processing in the same way as when combined with APP-wt. Furthermore, in protein constructs being degraded in a predetermined product line (SPA4/ 1-51, SPA4/1-52) G33A had the same strong effect on the consecutive processing. This implies that G33A particularly affects γ -cleavages rather than the primary ϵ -cleavage step. This also indicates that G33A exclusively influences processing within the A β 42 line.

In the presence of PS1-FAD mutations, the impact of G33A on A β generation was diminished, which might be attributable to the possible inhibition of substrate flux by PS1-FAD mutations. Thus, the mutation G33A acts downstream of APP-FAD mutations but only partially downstream of PS1-FAD mutations.

In addition to this data, a product-precursor relationship of A β 42 and A β 38 was indicated by the effects on A β production by (i) nonsteroidal anti-inflammatory drugs or γ -secretase modulators (41), (ii) several γ -secretase inhibitors (42), (iii) N-terminal elongation of pen-2 (43), and (iv) GxxxG mutations (9) as well as direct detection (V) of the tetrapeptide $V^{39}VIA^{42}$ arising from the A β 42 cleavage that generates AB38 (15). Page et al. (34) and Czirr et al. (44) concluded from their work that A β 42 and A β 38 are not related in their production as in the presence of PS1-FAD mutants nonsteroidal anti-inflammatory drugs sulindac sulfide and fenofibrate only had an attenuated effect on A β 38 and A β 42 levels. In agreement with this, we found only a tendency of G33A to change the A β 38/A β 42 levels in the presence of PS1-FAD mutants. We assumed that the attenuated effects are attributable to the inhibition of substrate flux by PS1-FAD mutations. Concordantly, we suggested previously that nonsteroidal anti-inflammatory drugs might act by modulating the substrate dimer stability (9), which recently has been supported by the finding that nonsteroidal anti-inflammatory drugs are substrate-targeted modulators, which possibly bind to the A β sequence (45).

CONCLUSION

APP-FAD and PS1-FAD mutations act differently on A β 42/ A β 40 production. Mechanistically, the analyzed familial mutations can be divided into three subgroups: (i) APP-FAD mutants that increase A β 42 and A β 38, (ii) APP-FAD mutants that decrease A β 40, and (iii) PS1-FAD mutants that increase A β 42, but decrease A β 38 levels. In the early steps of APP processing, APP-FAD mutations decide on the affiliation to the one or the other product line, and subsequently, the mutation G33A affects processing within the A β 42 line.

An impairment of the GxxxG-mediated dimerization of APP was sufficient to rescue the pathological processing effects of APP-FAD mutants. On average, 60% less A β 42 and 3-fold more A β 38 were produced by the APP-FAD-G33A mutants. The effects of G33A were found attenuated with PS1-FAD mutants attributable to the different pathogenic mechanism of PS1-FAD. Thus, our data supports the idea that the APP GxxxG motif represents a new drug target site not only for sporadic AD but also for early onset FAD.

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