

Qualitative changes in human γ -secretase underlie familial Alzheimer's disease

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Presenilin (PSEN) pathogenic mutations cause familial Alzheimer's disease (AD [FAD]) in an autosomal-dominant manner. The extent to which the healthy and diseased alleles influence each other to cause neurodegeneration remains unclear. In this study, we assessed γ -secretase activity in brain samples from 15 nondemented subjects, 22 FAD patients harboring nine different mutations in *PSEN1*, and 11 sporadic AD (SAD) patients. FAD and control brain samples had similar overall γ -secretase activity levels, and therefore, loss of overall (endopeptidase) γ -secretase function cannot be an essential part of the pathogenic mechanism. In contrast, impaired carboxypeptidase-like activity (γ -secretase dysfunction) is a constant feature in all FAD brains. Significantly, we demonstrate that pharmacological activation of the carboxypeptidase-like γ -secretase activity with γ -secretase modulators alleviates the mutant PSEN pathogenic effects. Most SAD cases display normal endo- and carboxypeptidase-like γ -secretase activities. However and interestingly, a few SAD patient samples display γ -secretase dysfunction, suggesting that γ -secretase may play a role in some SAD cases. In conclusion, our study highlights qualitative shifts in amyloid- β (A β) profiles as the common denominator in FAD and supports a model in which the healthy allele contributes with normal A β products and the diseased allele generates longer aggregation-prone peptides that act as seeds inducing toxic amyloid conformations.

Early-onset familial Alzheimer's disease (AD [FAD]), starting before age 65, is mainly caused by mutations in the *Presenilin 1/2* (*PSEN1/2*) or the *amyloid precursor protein* (*APP*) genes and represents less than 0.1% of the total AD cases (Campion et al., 1999). Although rare, FAD offers a unique model to gain insights into the molecular mechanisms and etiology of sporadic AD (SAD).

PSEN is the catalytic subunit of the γ -secretase complex (De Strooper et al., 1998; Wolfe et al., 1999), an intramembrane multimeric protease involved in the processing of many type 1 transmembrane proteins; among them, the Notch receptors and APP have received much attention be-

cause of their association with crucial cell signaling events or with AD pathogenesis, respectively (for a review see Jurisch-Yaksi et al. [2013]). Nicastrin (Nct), PSEN enhancer 2 (Pen2), and anterior pharynx defective 1 (APH1) are, together with PSEN, essential components of the protease complex (De Strooper, 2003).

More than 150 pathogenic mutations in *PSEN1* have been reported so far (<http://www.molgen.ua.ac.be/ADMutations>); and notably, the vast majority are missense substitutions distributed throughout the primary structure of PSEN1. PSEN/ γ -secretase hydrolyzes peptide bonds in a process called regulated intramembrane proteolysis, which allows translation of extracellular signals into the cell. Compelling evidence indicates that γ -secretase cuts membrane proteins sequentially: the first endopeptidase cleavage (e) releases a soluble intracellular domain (ICD), which may

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Abbreviations used: A β , amyloid- β ; AD, Alzheimer's disease; APP, amyloid precursor protein; DRM, detergent-resistant membrane; FAD, familial AD; GSM, γ -secretase modulator; ICD, intracellular domain; LOF, loss-of-function; Nct, Nicastrin; PSEN, Presenilin; SAD, sporadic AD.

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translocate to the nucleus to regulate gene expression while the remaining N-terminal transmembrane domain (TMD) fragment is successively cut by the carboxypeptidase-like activity of γ -secretase (γ -cleavages). Endopeptidase products, either amyloid- β 49 (A β 49) or A β 48, are then processed along two major product lines: A β 49 \rightarrow A β 46 \rightarrow A β 43 \rightarrow A β 40 or A β 48 \rightarrow A β 45 \rightarrow A β 42 \rightarrow A β 38 (Takami et al., 2009). Every γ -cleavage removes a short C-terminal peptide from the TMD, reducing its hydrophobicity and increasing the probability of release. Secretion of an N-terminal fragment into the extracellular/luminal space terminates this sequence (Qi-Takahara et al., 2005; Yagishita et al., 2008; Takami et al., 2009). Importantly, the efficiency of the endopeptidase cleavage determines ICD product levels, which acquires high physiological relevance in the case of the Notch substrate. The carboxypeptidase-like efficiency, the number of cuts per substrate, determines the length of the N-terminal products; the level of efficiency is pathologically very relevant in the case of the APP substrate, as lower efficiency results in the production of longer and more aggregation-prone A β peptides (Chávez-Gutiérrez et al., 2012).

How mutations in the PSENs cause FAD remains a hotly debated topic in the field. Because FAD is an autosomal-dominant disorder (patients carry both healthy and mutant alleles), a major unknown in the discussion remains the role of the healthy allele and, to a lesser extent, the role of the brain environment on the total (normal + mutant proteases) γ -secretase activity. To what degree do normal and mutant complexes contribute to total γ -secretase activity in the patient brain? Does the healthy allele compensate for the disease allele effects? Despite their relevance, those questions have not been addressed.

Only one group, i.e., Potter et al. (2013), have estimated the A β production kinetics in the FAD brain by measuring isotope-labeled A β peptides in the cerebrospinal fluid of patients (stable isotope-labeled kinetics [SILK]). Feeding the in vivo metabolic labeling patient data into a mathematical model, specifically generated for their approach, they described higher A β 42 production rates in the central nervous system of *PSEN* mutation carriers (Potter et al., 2013). Accordingly, Potter et al. (2013) suggest that increments in A β 42 play a decisive pathogenic role in AD.

In contrast, a “revised” loss-of-function (LOF) hypothesis has recently been proposed by Xia et al. (2015). In this view, loss of PSEN/ γ -secretase physiological cell signaling function causes neurodegeneration, whereas changes in A β peptides are only secondary byproducts that arise from but do not trigger the disease (Xia et al., 2015). The idea is only tenable if FAD-linked PSEN mutations exert a LOF effect on PSEN/ γ -secretase and, in addition, a dominant-negative effect on the healthy *PSEN* allele (normal γ -secretase) in patients, a key part of this hypothesis (Heilig et al., 2013; Xia et al., 2015). However, it should be stressed that γ -secretase haploinsufficiency caused by nonsense, frameshift, and splice site mutations in genes coding for essential subunits of γ -secretase

(Nct, Pen2, and PSEN) is pathogenic in nature; such haploinsufficiency causes a chronic inflammatory disease of hair follicles known as familial acne inversa. Most importantly, no clinical association between this disorder and AD has been reported (for a review see Pink et al. [2013]). Furthermore, if FAD-linked PSEN mutations were truly LOF mutations, resulting in “inactive” γ -secretase complexes, homozygous individuals for the disease allele would not be viable because of disturbances in Notch signaling during embryonic development. However, six individuals with homozygous *PSEN1 E280A* gene mutation have been identified (Kosik et al., 2015).

An alternative view to both hypotheses is that pathogenic mutations in *PSEN* cause disease by qualitative shifts in A β profile production (γ -secretase dysfunction; Chávez-Gutiérrez et al., 2012). We have demonstrated that loss of endopeptidase activity is not necessarily observed in γ -secretase complexes containing PSEN1/2 FAD-linked mutations, but reduced carboxypeptidase-like efficiency (γ -secretase dysfunction) is the constant denominator. Furthermore, FAD PSEN mutations may affect the carboxypeptidase-like γ -secretase activity at multiple turnovers, resulting in increased A β 43 and A β 42 levels as well as in other longer A β peptides, such as A β 45 and A β 46 (Quintero-Monzon et al., 2011; Chávez-Gutiérrez et al., 2012; Fernandez et al., 2014). These data support a model in which relative, rather than absolute, changes in A β product profiles are at the basis of PSEN/ γ -secretase-mediated pathogenicity. However, these findings were based on studies conducted in PSEN1/2-deficient MEFs, which does not fully recapitulate the in vivo heterozygous situation in the FAD patient's brain.

In the current study, we investigated processing of APP by the γ -secretase complex in postmortem human brain samples from FAD and SAD patients and healthy control subjects. Our investigation is the first to directly assess γ -secretase activity in brain material from FAD mutant carriers and to address how the FAD-linked mutant heterozygous situation in patients affects γ -secretase function in brain.

RESULTS AND DISCUSSION

A β production rates in FAD and SAD brains

We aimed to evaluate the effects of pathogenic PSEN1 mutants on total γ -secretase activity (healthy and disease *PSEN1* alleles) in human brain samples from FAD patients. As a first step, we sought to determine and contrast the production rates of A β peptides in (a) human control brains, i.e., containing two healthy *PSEN1* alleles; (b) FAD brains carrying pathogenic mutations in *PSEN1*, heterozygous for the *PSEN1* alleles; and (c) SAD brains, with two healthy *PSEN1* alleles. A β peptides are generated from APP-C99 membrane peptide by consecutive γ -secretase proteolytic cleavages. The first endopeptidase cut (e) releases a soluble ICD (AICD) and generates a long membrane-associated A β peptide, either A β 49 or A β 48, which are then processed along two major product lines (Takami et al., 2009).

Active γ -secretase is associated with detergent-resistant membranes (DRMs; Wahrle et al., 2002) and DRMs pre-

Table 1. Clinical data of FAD patients whose brains were analyzed in this study

Mutation	Sex	Diagnosis	Age of onset	Age at death	APOE
			yr	yr	
V89L	M	FAD	48	57	23
Intron 4	F	FAD	35	51.9	44
Intron 4	F	FAD	36	41.6	33
E120G	M	FAD	34	44	33
M139T	M	FAD	47	64	33
M139T	M	FAD	48	57	33
M139T	M	FAD	45	53	33
I202F	F	FAD	48	59.3	44
P264L	F	FAD	45	56	44
P264L	M	FAD	53	60	34
R278I	F	FAD	46	65.6	34
L286P	F	FAD	35	56	33
E280A	F	FAD	47	54	33
E280A	M	FAD	44	52	33
E280A	M	FAD	54	63	34
E280A	M	FAD	47	56	33
E280A	F	FAD	46	67	34
E280A	F	FAD	48	64	33
E280A	F	FAD	43	48	33
E280A	F	FAD	50	60	33
E280A	F	FAD	52	68	33
E280A	M	FAD	47	58	33

pared from brain or cells are a bona-fide source of γ -secretase activity (Matsumura et al., 2014). Thus, we prepared DRMs from the prefrontal cortices of 15 control brain samples, 22 FAD brain samples carrying nine different pathological PSEN1 mutations, V89L (1 case), intron 4 (2 cases), E120G (1 case), M139T (3 cases), I202F (1 case), P264L (2 cases), R278I (1 case), E280A (10 cases), and L286P (1 case; Table 1); and 11 SAD brain samples. DRMs were used as a source of the enzyme in *in vitro* activity assays. Importantly, DRMs contain γ -secretase in its native environment (the membrane) while maintaining the lipid composition of their origin (cells or brain).

The production rates of the A β 38, A β 40, and A β 42 peptides (*de novo*) were determined by incubation of equivalent amounts of brain DRMs with the purified APPC99-3 \times FLAG γ -secretase substrate, under saturating conditions for 0 and 4 h. Our results show a reduction in the A β 38 production rate in the majority of FAD cases and a reduction in A β 40 generation in five of nine FAD cases (Fig. 1, A and B). In contrast, A β production in SAD samples did not show significant alterations (Fig. 1, A–C). However, the dispersion observed in the SAD group may be an indication of changes in γ -secretase activity in a fraction of late-onset AD cases (see next section for further discussion). Most interestingly, there were no differences in A β 42 production rates in most of the FAD brain samples, except for the intron 4 mutation cases, which display, on average, a 2.5-fold increment over controls (Fig. 1 C). A mild reduction in A β 42 production rate was observed in the E120G-, P264L-, and R278I-PSEN1 brain samples, but the differences did not reach statistical significance. The γ -secre-

tase inhibitor X (a transition state analogue) abolished the production of A β peptides (not depicted), demonstrating the specificity of the reaction. Unfortunately, the concentration of “*de novo*” generated A β 37 and A β 43 peptides in control and most of the mutant samples were below the detection limits.

To correct for any potential differences in protein concentrations in our assays, we also normalized A β production to flotilin-1 levels, determined by immunoblot, which did not change our observations (not depicted). Collectively, our data support a model in which relative changes in A β production in FAD are more important for disease than absolute increments in A β 42 levels (Tanzi and Bertram, 2005; De Strooper, 2007; Kuperstein et al., 2010; Chávez-Gutiérrez et al., 2012). Thus, our findings contrast with those of Potter et al. (2013). The differences may arise from the fact that the SILK method measures A β released in the interstitial fluid, which provides an indirect and perhaps not so accurate assessment of γ -secretase activity in human brain. Although their mathematical model should in principle correct for these potential issues, several not yet experimentally verified assumptions were made with regard to production, secretion, and clearance mechanisms of A β peptides (for further discussion of the model see Edland and Galasko [2011]). For instance, it is assumed that different A β s are generated independently from each other (Potter et al., 2013), which is not in line with the current knowledge showing that consecutive γ -secretase cleavages generate A β peptides (Takami et al., 2009). Furthermore, we would like to draw attention to the heterogeneous behavior of the mutation carrier cohort, reported as a proof of concept in Potter et al. (2013): the A β 42 production rates were actually only elevated in three out of seven FAD-linked PSEN1 mutation carriers.

De novo production of AICD in FAD-PSEN1 brain samples

Our results indicate that A β 38, A β 40, and A β 42 are the main secreted products, and the sum of “*de novo*” A β 38, A β 40, and A β 42 products reveals lower A β production in brain samples carrying pathogenic mutations in PSEN1 in five out of nine cases, relative to control and SAD cases (Fig. 1 D). The observed effect on A β production could be caused by a reduction in the endopeptidase activity or an impaired carboxypeptidase-like efficiency in FAD brain samples. Therefore, we analyzed the production of AICD in our samples, which provides a relative indication for the efficiency of the γ -secretase endopeptidase activity in the tested human brain samples. Decreased γ -secretase endopeptidase efficiency may result in alterations in cell signaling events involved in cellular communication (LOF). We found no significant differences in the production rates of AICD (*de novo* AICD) in controls and FAD brain samples, although a slight reduction in AICD production rate was observed in the R278I brain sample (Fig. 1, E and F). These data clearly indicate that the overall γ -secretase endopeptidase activity is unaffected in most of the FAD brain samples, and therefore, this effect cannot be an essential part of the pathogenic mechanism.

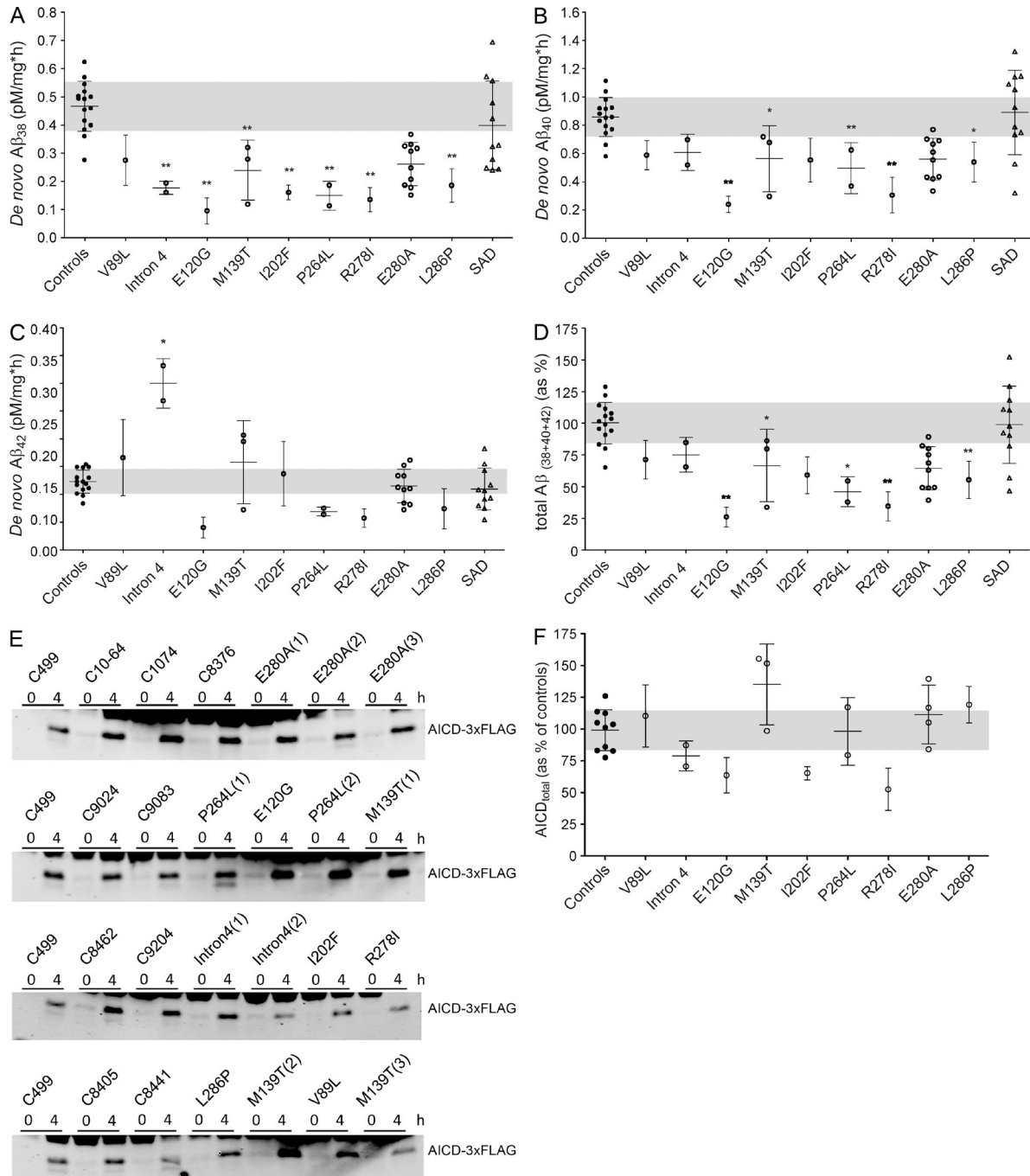


Figure 1. **APP processing in human brain samples from FAD, SAD, and control patients.** (A–D) Aβ production rate in brains of FAD or SAD patients compared with nondemented subjects. To determine de novo production of Aβ peptides, CHAPSO-resistant membranes prepared from brain tissue of patients were incubated with 1.5 μM C99-3×FLAG substrate and quantified using MSD ELISA technology. Graphs show mean ± SD for groups with one case or mean of means ± SD for groups with number of cases greater than one. (E) SDS-PAGE/Western blot showing AICD product levels in reactions with human control and FAD brains. The molecular mass of AICD-3×FLAG is ~10 kD. (F) De novo AICD product levels (endopeptidase activity levels) in human control and FAD brain samples. Graph shows mean ± SE for groups with one case or mean of means ± SE for groups with number of cases greater than one. All experiments were repeated at least three times, and statistical significance was tested with one-way ANOVA and Dunnett's post test, taking the corresponding WT set as the control group (**, $P < 0.01$; *, $P < 0.05$).

We have previously shown that pathogenic PSEN1 mutants display variable effects (including no effect) on the endopeptidase efficiency of mutant γ -secretase complexes but consistently reduce the γ -secretase carboxypeptidase-like efficiency (γ -secretase dysfunction), all relative to the normal enzyme (Chávez-Gutiérrez et al., 2012). To evaluate the effects of the pathogenic mutations analyzed here on γ -secretase activity, we stably expressed the different clinical mutant PSENs in a *Psen1/2* knockout background (Fig. 3). With the exception of the R278I PSEN1 mutant, which severely impairs activation of the γ -secretase complex, *Psen1/2* knockout MEFs transduced with normal or mutant PSEN1s express comparable levels of the mature γ -secretase complex (Fig. 3 A). Specific endopeptidase activities, defined as “de novo AICD” normalized against PSEN1-CTF subunit levels in the *in vitro* reactions, revealed variable effects (including no effect) of the PSEN1 mutations on the endopeptidase function of γ -secretase (Fig. 3 B). Interestingly, comparison of the endopeptidase activities of samples containing either normal or mutant complexes (Fig. 3 B) with FAD brain samples (normal + mutant enzymes; Fig. 1 F) demonstrates that the healthy allele (normal enzyme) compensates for (if any) decrements in the endopeptidase cleavage rates caused by the disease allele (mutant γ -secretase complex). The above implies that normal and mutant proteases contribute to γ -secretase endopeptidase activity. Undoubtedly, our data do not support a mutant-mediated “dominant-negative effect” on the healthy allele and, therefore, contrast with the hypothesis proposed recently by Heilig et al. (2013) and Xia et al. (2015).

Carboxypeptidase-like efficiency in FAD and SAD brain samples

Interestingly, brain samples carrying pathogenic PSEN1 mutations consistently display lower “A β 38 + A β 40 + A β 42” production rates (Fig. 1 D) than for AICD (Fig. 1 F), suggesting a higher production of longer A β peptides (>A β 42) in FAD versus control brain samples, which may be indicative of impaired carboxypeptidase-like efficiency (γ -secretase dysfunction). γ -Cleavage efficiency can be assessed by determining the A β 38/A β 42 ratio, which represents the product/substrate ratio for the fourth catalytic turnover of the γ -secretase. Significantly, changes in this ratio correlate directly with this particular cleavage efficiency. Thus, we calculated the A β 38/A β 42 ratios of total γ -secretase in FAD, SAD, and control brain samples. In the FAD brain samples, the observed decrement in the short A β 38 and A β 40 peptides translated into a significant reduction in the A β 38/A β 42 ratio (Fig. 2 A). Remarkably, regardless of the nature and position of the mutation in PSEN1, the A β 38/A β 42 ratios revealed a consistent reduction in total γ -secretase carboxypeptidase-like efficiencies in FAD brain samples, relative to controls. Our data thus show no alterations in the total (normal + mutant complexes) γ -secretase endopeptidase activity and lower de novo production of A β 38 and A β 40 peptides. This is not accompanied by increased A β 42 production levels in

most of the FAD patient brain samples. These results strongly suggest that longer A β peptides (such as A β 43 and A β 45) are produced in patient brain samples. Unfortunately, technical limitations do not allow us currently to measure the production of these longer peptides.

The amplitude of the effects on the A β 38/A β 42 ratios did not correlate with the age at onset (Table 1), suggesting that additional genetic and/or environmental factors may play a role in the onset of FAD, and we speculate that altered processing of other substrates could contribute to this. We also looked at the A β 42/A β 40 ratio because increments in this (somewhat deliberately chosen) ratio have been used as hallmark of FAD mutations for decades. Our data show increased A β 42/A β 40 ratios in six (V89L, intron 4, M139T, I202F, R278I, and E280A) of the nine PSEN1 mutant cohorts analyzed.

With regard to SAD, our data reveal similar γ -secretase carboxypeptidase-like efficiencies relative to control brain samples. However, in accordance with the A β production velocities (Fig. 1, A–D), we observed a marked A β 38/A β 42 ratio dispersion among the SAD brain samples (Fig. 2 A). This may indicate alterations in γ -secretase carboxypeptidase-like efficiency in a subset of late-onset SAD cases but exclude the hypothesis that changes in γ -secretase efficiency play a major role in the majority of SAD patients. Application of *in vivo* metabolic labeling in SAD patients found no changes in A β production in late AD (Mawuenyega et al., 2010). However, analysis of other γ -secretase products in cerebrospinal fluid of late-onset patients revealed the apparent existence of subpopulations of SAD patients showing differential alterations in γ -secretase activity, including enzyme dysfunction (Hata et al., 2011, 2012). In addition, increments in the carboxypeptidase-like efficiency of γ -secretase in SAD brain samples have also been reported (Kakuda et al., 2012). Thus, our data support the idea that the causes of late-onset AD are heterogeneous and raise the possibility that SAD patients showing γ -secretase dysfunction could benefit from γ -secretase activation (see next section). However, the analysis of a larger cohort is needed to accurately evaluate the relevance of γ -secretase dysfunction in the sporadic form of AD.

γ -Secretase modulators (GSMs) alleviate the FAD-associated effects of the majority of PSEN1 mutations

GSMs act as activators of the carboxypeptidase-like activity of the γ -secretase complex (Chávez-Gutiérrez et al., 2012; Takeo et al., 2014). Given the observed reduction in the γ -secretase carboxypeptidase-like efficiency in FAD patient brain samples, we hypothesized that incubation with GSMs would enhance the carboxypeptidase-like activity and thereby correct for the pathogenic A β profiles associated with FAD. Thus, we decided to investigate the effects of two different GSM families on the efficiency of the γ -secretase carboxypeptidase-like activity in FAD brain samples. Specifically, we tested an acid- and an imidazole-based modulator at 1 μ M in our *in vitro* reactions (Fig. 2, B and C, respectively). Fig. 2

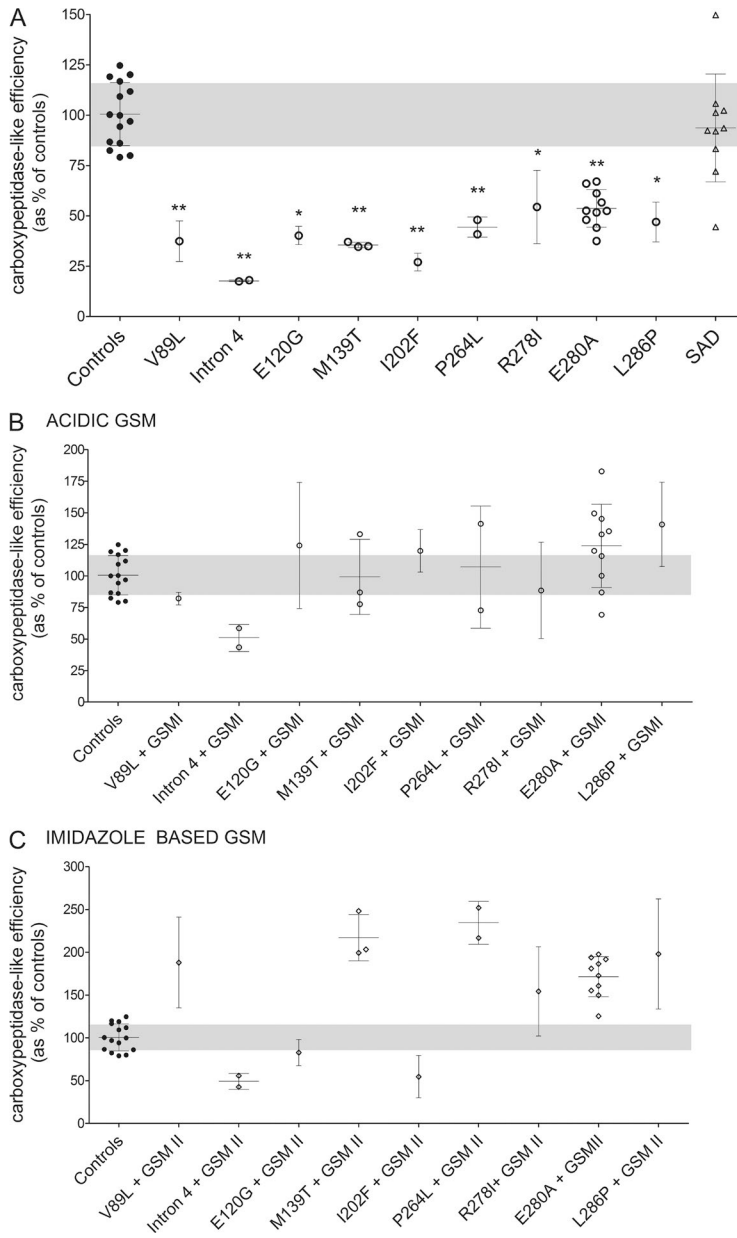


Figure 2. AD-causing PSEN1 mutants impair γ -cleavage efficiency in FAD human brain samples, and GSMs correct for the pathogenic effect. (A) Carboxypeptidase-like efficiency seen as $A\beta_{38}/A\beta_{42}$ (product/substrate) ratio. (B and C) The responses to GSMs observed in mutation carrier brain samples is contrasted with the carboxypeptidase-like efficiencies measured in brain samples from nondemented subjects (gray area, shown in panel A). To determine the response to GSMs, CHAPS0-resistant membranes prepared from brain tissue of patients were incubated with $1.5 \mu\text{M}$ C99-3 \times FLAG substrate in the presence of $1 \mu\text{M}$ GSM. Graphs show mean \pm SD for groups with one case or mean of means \pm SD for groups with number of cases greater than one. All experiments were repeated three to five times, and statistical significance was tested with one-way ANOVA and Dunnett's post test, taking the corresponding WT set as the control group (**, $P < 0.01$; *, $P < 0.05$).

(B and C) shows that both GSMs restore the efficiency of the carboxypeptidase-like activity to control levels in eight out of nine FAD PSEN1 and seven out of nine FAD PSEN1 brain samples, respectively. Patient brain samples carrying the intron 4 mutation displayed limited responses to both GSMs. Similarly, the imidazole-based GSM did not restore the efficiency of the carboxypeptidase-like activity in the I202F case. The first extracellular loop of PSEN is part of an allosteric ligand-binding site within the N-terminal fragment of PSEN (Takeo et al., 2014); most likely, the intron 4 and I202F mutations disrupt the binding of GSMs to PSEN/ γ -secretase. These data indicate that GSM treatment may be particularly useful in FAD, although the magnitude of the modulatory response may depend on the nature of the mutation and the

GSM chemistry. A very recent study reached similar conclusions on the effects of GSMs on FAD, using as a model neuronal cultures derived from FAD patient induced pluripotent stem cells (Moore et al., 2015). We would like to point out that full documentation of GSM-mediated effects on $A\beta$ profiles should be performed before any clinical applications. In particular, potential modulatory effects on the generation of long $A\beta$ peptides (>42 amino acids) should be considered to prevent undesired alterations at that level.

Collectively, these studies demonstrate no significant differences in the endopeptidase activity levels between FAD and control brain samples. The lack of effect on AICD production ascertains that the effects on the $A\beta_{38}/A\beta_{42}$ ratio are not simply caused by the extensive damage in the late-

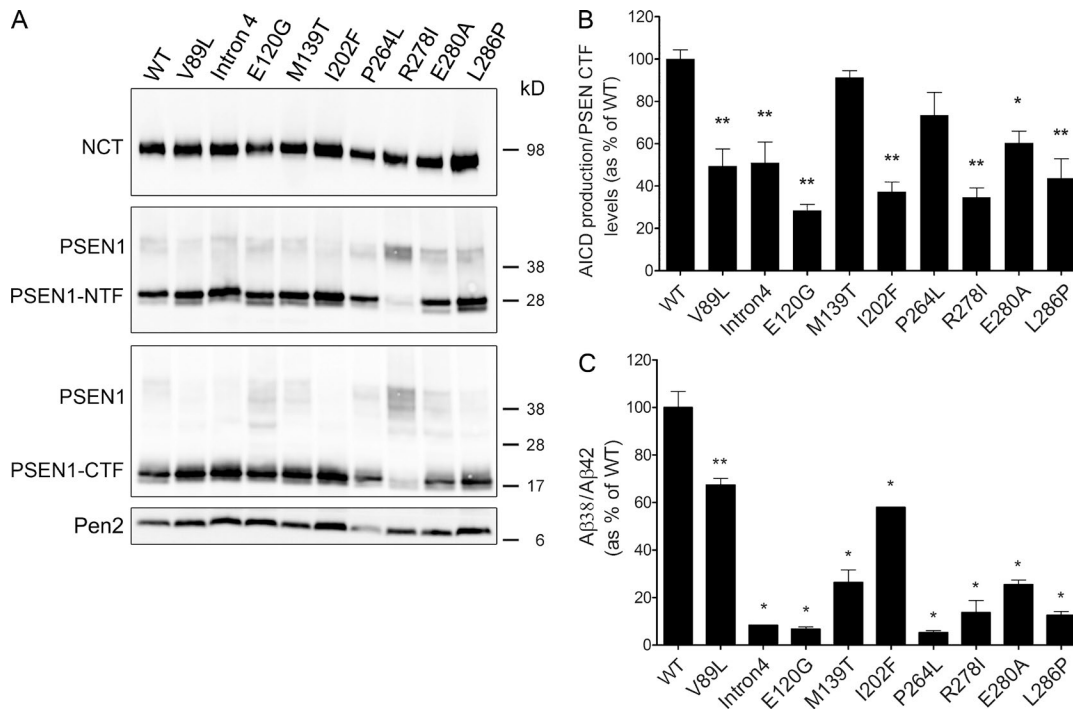


Figure 3. **FAD-PSEN1 mutations show variable effects on the endopeptidase cleavage, but all impair the fourth enzymatic turnover of γ -secretase.** (A) Nct, PSEN1-NTF, PSEN1-CTF, and Pen2 protein levels in *Psen1/2*^{-/-} MEFs transduced to express human WT or FAD-PSEN1. (B) Measurement of AICD production for WT and mutant PSEN1 γ -secretase complexes. To determine specific activities for WT and FAD complexes, AICD products were normalized to PSEN1-CTF fragment levels quantified by Western blot. (C) FAD-PSEN1 mutations consistently impair the fourth catalytic cleavage seen as A β 38/A β 42 (product/substrate) ratio, relative to WT activity. All experiments were repeated three to five times. Graphs show mean \pm SE, and statistical significance was tested with one-way ANOVA and Dunnett's post test, taking the corresponding WT set as the control group (**, $P < 0.01$; *, $P < 0.05$).

stage AD brains. Interestingly, when expressed in homozygous *Psen1/2*-deficient MEFs, some of the clinical mutants cause by themselves decrements in the overall γ -secretase endopeptidase activity (seven mutations cause a reduction ranging from 70% to ~30% of the normal γ -secretase activity, and two mutations did not affect activity; Fig. 3 B). The presence of the normal *PSEN1* allele in heterozygous FAD patients probably compensates for the decrease in γ -secretase endopeptidase activity observed for some of the pathogenic PSEN1 mutants in homozygous *Psen1/2*-deficient MEFs (Fig. 3 B). Thus, our investigation does not support a dominant-negative effect of the FAD allele over the healthy allele (normal PSEN1/ γ -secretase), as proposed by others (Xia et al., 2015). However, we cannot discard the proposition that misprocessing of other γ -secretase substrates may contribute to disease symptoms (discussed in Chávez-Gutiérrez et al. [2012]) and help to explain the wide clinical spectrum observed in FAD patients (reviewed in Bergmans and De Strooper [2010]).

Significantly, this study is important in its demonstration that γ -secretase dysfunction is the common denominator in FAD patient brain samples (Fig. 2 A). These findings are entirely consistent with the effect of FAD-linked PSEN mutations on γ -secretase function shown in Fig. 3 C and in our previous work (Chávez-Gutiérrez et al., 2012). Our data

highlight that qualitative shifts in A β product profiles, toward longer A β peptides, are the central feature in FAD pathogenesis, although elevated A β 42 peptide could contribute to pathogenesis in some FAD cases.

The qualitative shifts in the A β profiles observed with the clinical mutations suggest that longer A β peptides (≥ 42) may promote neurotoxicity by providing the seeds for "toxic oligomers," even at low concentrations. In this regard, a recent publication has put emphasis on the high amyloidogenicity and pathogenicity of A β 43 (Saito et al., 2011). Although we could not quantify this peptide in our tests on brain samples, because the amounts generated did not reach the threshold of detection, our previous work with membranes from *Psen1/2*-deficient fibroblasts expressing WT or mutant PSENs clearly indicates that FAD-linked mutants elevate the relative production of A β 43 (Chávez-Gutiérrez et al., 2012). Furthermore, we show that the A β 40 production rate is consistently low in FAD patient brain samples. Depletion of this particular peptide may also contribute to pathogenesis (Wang et al., 2006; Kim et al., 2007). Notably, minor changes in A β profiles have been reported to have drastic effects on neurotoxicity (Kuperstein et al., 2010). A phase II clinical trial using a humanized antibody against different A β 42 assemblies (crenezumab; Adolfsson et al., 2012) is currently being

conducted on PSEN1-E280A patients in Colombia (<http://www.clinicaltrials.gov/ct2/show/NCT01998841>). According to the observed γ -secretase dysfunction in FAD, it would be of relevance to test the affinity of crenezumab for other long, aggregation-prone A β peptides.

With regard to SAD, our data reveal a heterogeneous group in terms of γ -secretase activity, suggesting that subpopulations of late-onset patients may present alterations in A β production, which could be of relevance for future “personalized” treatment strategies. However, the fact that most patients do not show altered γ -secretase activity supports the view that accumulation of A β peptides in the central nervous system of SAD patients is more frequently caused by impaired A β peptide clearance (Mawuenyega et al., 2010). The potential causes of alterations in γ -secretase function in these few late-onset cases are intriguing, and the implications of such changes on disease onset, progression/duration, and therapy are currently unknown.

In conclusion, our investigation is the first to assess how the heterozygous situation in patients actually affects γ -secretase function in human brain. We find no evidence for a loss of overall γ -secretase endopeptidase function. Alternatively, we propose that qualitative changes in A β product profiles are the basis of PSEN/ γ -secretase-mediated pathogenicity. These findings imply that long A β peptides are potently pathogenic, and we speculate that a small alteration in the clearance of these long amyloidogenic peptides may contribute to late-onset AD. Finally, our findings may have direct implications in therapy, as they indicate that activation of the carboxypeptidase-like activity (while respecting the endopeptidase function) could be a promising therapeutic concept in FAD.

MATERIALS AND METHODS

Antibodies and reagents. Antibodies were purchased as follows: MAB5232 against human PSEN1-CTF from EMD Millipore, 18189 rabbit polyclonal against human Pen2 from Abcam, 612290 and 610820 mouse monoclonal anti-human NCT and anti-human flotillin-1 from BD. ELISA antibodies and GSMs were obtained through collaboration with Janssen Pharmaceutica NV, Beerse, Belgium: JRF AB038 for A β 1-38, JRF/cAb40/28 for A β 1-40, JRF/cAb42/26 for A β 1-42, and detection antibody JRF/AbN/25 against the N terminus of A β . Acid-based ((2-[(1R,2S)-1-[4-methyl-1-[4-(trifluoromethyl)phenyl]pentyl]-2-[4-(trifluoromethyl)phenyl]-4-piperidyl] acetic acid) and imidazole-based (*N*-[2-fluoro-5-(trifluoromethyl)phenyl]-5-[3-methoxy-4-(4-methylimidazol-1-yl)phenyl]-2-methyl-1,2,4-triazol-3-amine) GSMs were synthesized according to described procedures (Crump et al., 2011; Velter et al., 2014). γ -Secretase inhibitor refers to inhibitor X purchased from EMD Millipore.

Expression and purification of C99-3 \times FLAG substrate. Substrate expression and purification was performed as previously described (Chávez-Gutiérrez et al., 2008). Purity was

assessed by SDS-PAGE and Coomassie staining (gelcode reagent; Thermo Fisher Scientific).

Subjects. Human cortical specimens for quantification of γ -secretase activity were obtained from Brain Bank at Tokyo Metropolitan Institute of Gerontology, Queen Square Brain Bank for Neurological Disorders at University College London, throughout collaboration with the Neuroscience Group of Antioquia Brain Bank at University of Antioquia, Medellín, Colombia, and the Neurological Tissue Bank of the Biobanc-Hospital Clinic-IDIBAPS. All of the samples came from brains that were removed and placed in -80°C within 65 h postmortem (patients were moved to a cold room within 2 h after death). Samples were collected according to protocols approved by respective ethical boards, and written legal consents for the use of organs for medical research are available for each patient. A total of 48 brain samples were used for the reported project: 6 SAD cases and 10 controls from the Brain Bank at Tokyo (Brodmann areas 9–11); 4 different PSEN1 FAD mutations from the Queen Square Brain Bank at University College London (1 patient per mutation, Brodmann areas 9–11); 10 E280A FAD cases, 5 SAD cases, and 4 controls from D. Sepulveda-Falla (Brodmann area 11); 5 different PSEN1 FAD mutations (8 patients, Brodmann areas 9–11; Pera et al., 2013); and 1 control from the Neurological Tissue Bank of the Biobanc-Hospital Clinic-IDIBAPS. All human protocols were approved by Medical Ethics Committee UZ KU Leuven, Belgium.

Generation of MEFs. *Psen1/Psen2*^{-/-} MEFs (Herreman et al., 2000) were cultured in Dulbecco's modified Eagle's medium/F-12 (Life Technologies) containing 10% fetal bovine serum. MEFs were transduced using pMSCV-puro, a replication-defective recombinant retroviral expression system (Takara Bio Inc.) harboring cDNA inserts coding for WT human PSEN1 or variants:V89L, intron 4 (p.L113_I114insT), E120G, M139T, I202F, I213T, P264L, R278I, and L286P. Stable cell lines were selected using 5 $\mu\text{g}/\text{ml}$ puromycin (Sigma-Aldrich).

DRM preparation from human brains or MEFs. CHAPSO DRMs were prepared for human brain frontal cortices as previously described (Kakuda et al., 2012) with minor modifications or from MEFs expressing WT γ -secretase or mutant complexes containing PSEN1 mutations. In the first case, after careful removal of leptomeninges and blood vessels, <250 mg blocks of tissue were homogenized in ~ 10 vol of 10% sucrose in MBS buffer (25 mM MES, pH 6.5, 150 mM NaCl) containing 1% CHAPSO (Sigma-Aldrich) and protease inhibitors (Complete; Roche). In the case of MEFs, total membranes were prepared from 12 big culture dishes (245 \times 245 \times 25), and membrane pellets were homogenized in ~ 2.5 ml of 10% sucrose in MBS buffer (25 mM MES, pH 6.5, 150 mM NaCl) containing 1% CHAPSO and protease inhibitors. Each homogenate was mixed with equal volume of 70% sucrose in MBS buffer, and 4 ml was placed at the bottom of

an ultracentrifuge tube (344059; Beckman Coulter) and successively overlaid with 4 ml of 35% sucrose and 4 ml of 5% sucrose, both in MBS buffer. Samples were centrifuged at 39,000 rpm for 20 h at 4°C on an SW 41 Ti rotor (Beckman Coulter). After centrifugation, the DRM fraction (interface of 5%/35% sucrose) was carefully collected, rinsed in 20 mM PIPES, pH 7.0, 250 mM sucrose, and 1 M EGTA, and recentrifuged twice (100,000 g, 60 min, 4°C). The resultant pellet was resuspended with the aforementioned buffer using a 26G syringe and stored at -80°C until use. All DRM fractions used in this study were set to 1 µg/µl with 20 mM PIPES, pH 7.0, 250 mM sucrose, and 1 mM EGTA. Protein levels were tested by immunoblot using anti-flotillin-1 antibody.

Quantification of Aβ production rates by MSD ELISA. To determine de novo production of Aβ peptides, 6 µg CHA PSO-resistant membranes were incubated for 0 or 4 h at 37°C with 1.5 µM C99-3×FLAG substrate. The activity assays were performed in the presence of 2.5% DMSO (or 1 µM GSM in DMSO), 1 mM EGTA, 0.3% CHAPSO, and protease inhibitors (Complete; Roche). Aβ38, Aβ40, and Aβ42 levels in reactions were quantified on Multi-Spot 96-well plates precoated with anti-Aβ38, -Aβ40, and -Aβ42 antibodies using multiplex MSD technology. MSD plates were blocked with 150 µl/well 0.1% casein buffer for 1.5 h at room temperature (600 rpm) and rinsed 5× with 200 µl/well washing buffer (PBS + 0.05% Tween-20). 25 µl SULFO-TAG JRF/AbN/25 detection antibody diluted in blocking buffer was mixed with 25 µl of standards (synthetic human Aβ1-38, Aβ1-40, and Aβ1-42 peptides) or reaction samples diluted in blocking buffer and loaded 50 µl per well.

After overnight incubation at 4°C, plates were rinsed with washing buffer and 150 µl/well of the 2× MSD Read Buffer T (Tris-based buffer containing tripropylamine, purchased from Meso Scale Discovery) was added. Plates were immediately read on a Sector Imager 6000 (Meso Scale Discovery). We determined the rates at which Aβ38, Aβ40, and Aβ42 are produced in each sample by subtracting the 0-h value from the 4-h value obtained by ELISA (Meso Scale Discovery) and normalizing Aβ amounts against time to express rates in pM/h. To address the effects of modulators, we performed reactions in the presence of 1 µM GSMs. The ratio Aβ38/42 was taken as an estimation of the efficiency of the fourth turnover of the γ-secretase complex.

Statistical analysis. All statistical analysis was performed using Prism 6 software (GraphPad Software). An ANOVA test was used to test the significance of the changes between groups.

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