Destabilized γ-Secretase-Aβn interactions cause Alzheimer's disease, implications for drug discovery

Maria Szaruga[1,2], Bogdan Munteanu[3], Sam Lismont[1,2], Sarah Veugelen[1,2], Katrien Horré[1,2], Marc Mercken[4], Takaomi C. Saido[5], Natalie S. Ryan[6], Tatjana De Vos[7,8], Savvas N. Savvides[7,8], Rodrigo Gallardo[1,9], Joost Schymkowitz[1,9], Frederic Rousseau[1,9], Nick C. Fox[6], Carsten Hopf [3], Bart De Strooper[1,2,10,*] and Lucía Chávez-Gutiérrez[1,2,*]

[1]VIB Center for Brain & Disease Research, [2] KU Leuven, Department of Neurosciences, Leuven Institute for Neuroscience and Disease (LIND), 3000 Leuven, Belgium [3]Center of Applied Research in Biomedical Mass Spectrometry (ABIMAS), Mannheim University of Applied Sciences, 68163 Mannheim, Germany, [4]Janssen Research & Development, Division of Janssen Pharmaceutica NV, 2340 Beerse, Belgium, [5]Laboratory for Proteolytic Neuroscience, RIKEN Brain Science Institute, Japan, [6]Dementia Research Centre, Department of Neurodegenerative Disease, [7]Laboratory for Protein Biochemistry and Biomolecular Engineering, Department of Biochemistry and Microbiology, Ghent University and [8]VIB-UGent Center for Inflammation Research, Technologiepark 927, 9052 Ghent, Belgium, [9] Department of Cellular and Molecular Medicine, University of Leuven, Herestraat 49, 3000 Leuven, Belgium. [10]Dementia Research Institute UK, University College London, Queen Square, WC1N 3BG London, UK

***Correspondence:**

Lucia.ChavezGutierrez@cme.vib-kuleuven.be **or** Bart.DeStrooper@cme.vib-kuleuven.be

Summary

Alzheimer's disease (AD) linked mutations in Presenilins (PSEN) and the Amyloid Precursor Protein (APP) lead to production of longer amyloidogenic Aβ peptides. The shift in Aβ length is fundamental to the disease; however, the underlying mechanism remains elusive. Here, we show that substrate shortening progressively destabilizes the consecutive enzyme/substrate (E-S) complexes that characterize the sequential γ-secretase processing of APP. Remarkably, pathogenic PSEN or APP mutations further destabilize labile E-S complexes and thereby promote generation of longer Aβ peptides. Similarly, destabilization of wild type E-S complexes by temperature, compounds or detergent promotes release of amyloidogenic Aβ. Our work presents a unifying model for how PSEN or APP mutations enhance amyloidogenic Aβ production, suggests that environmental factors may increase AD risk, and provides the theoretical basis for the development of γ-secretase/substrate stabilizing compounds for the prevention of AD.

Introduction

. More than 200 mutations in Presenilin 1 or 2 (PSEN1/2) (*Enzyme*) (Sherrington et al., 1995) and about 20 in the Amyloid Precursor Protein (APP) (*Substrate*) (Goate et al., 1991) strongly support the relevance of Aβ (*Products*) in AD pathogenesis. PSEN1/2 are the catalytic subunits of distinct γ-secretase intramembrane protease complexes (De Strooper et al., 1998; Wolfe et al., 1999), which additionally contain Nicastrin (NCT), Presenilin-enhancer 2 (PEN2) and the Anterior pharynx defective 1 (APH1) as essential components (De Strooper and Chávez Gutiérrez, 2015).

Several studies point to long Aβ peptides ($\geq A\beta_{42/43}$) as key players in the initiation of aggregation of toxic Aβ-derived species, which ultimately lead to neurodegeneration in AD (Benilova et al., 2012; Haass and Selkoe, 2007). Thus, understanding the mechanisms that define the Aβ product length is highly relevant for the development of efficient strategies that target toxic Aβ production as part of AD disease-modifying therapy.

γ-Secretases generate Aβ peptides of different lengths from APP and AD causative PSEN mutations consistently decrease γ-secretase processivity (number of cuts per substrate molecule) shifting Aβ profiles towards longer and thereby more amyloidogenic peptides (Chávez-Gutiérrez et al., 2012; Fernandez et al., 2014). Although highly relevant, mechanistic understanding of γ-secretase function is very limited. For instance, we do not know how γ-secretase recognizes substrates or what drives the sequential cleavage of APP, or how clinical mutations in PSEN lead to the release of longer Aβ peptides. Such lack of fundamental insights has allowed controversies about the pathogenic role of PSEN (Veugelen et al., 2016) to grow (for discussion see De Strooper and Chavez, 2015).

Recent structural studies on γ-secretase (Bai et al., 2015a, 2015b) (**Figure S1A**) reveal that PSEN adopts a loosely organized, likely metastable fold, that co-exists in several conformations. These findings are

in line with previous low resolution structural analyses (Elad et al., 2014; Li et al., 2014), and with FRET-based studies presenting the PSEN/ γ-secretase complex as a dynamic entity (Lleo et al., 2004; Uemura et al., 2009, 2010; Wahlster et al., 2013).

Interestingly, elegant studies depict the rhomboid intramembrane proteases also as intrinsically metastable proteolytic systems (Baker and Urban, 2012). Given that protein stability results from a cooperative network of weak interactions, scattered distributed mutations throughout a structure can destabilise the entire native fold (Freire, 1999). If PSEN structure, as rhomboids, relied on a network of weak interactions throughout the molecule, one could hypothesize that scattered FAD-linked PSEN mutations might further "destabilize" its metastable fold and could impact protease function.

γ-Secretase displays a complex proteolytic activity (Takami et al., 2009). An initial ε-endopeptidase cleavage releases the soluble intracellular domain (AICD) and generates a long Aβ fragment (either $A\beta_{49}$ or $A\beta_{48}$) that is successively cut by carboxypeptidase-like γ-cleavages generating shorter $A\beta_n$ peptides (**Figure 1A**) until Aβ release stops the process. Accordingly, the sequential processing of APP by γ-secretase involves the formation of distinct enzyme-substrate (E-S) complexes, with each of them containing shortened *de novo* $A\beta_n$ substrates.

We hypothesized that the stability of each E-S complex determines the probability of its dissociation and thereby the length of Aβ peptides released (Aβ profiles). We thus performed thermo-activity assays to quantitatively evaluate the stability of E-S complexes. and find that these become progressively less stable with shortening of the consecutive $A\beta_n$ substrates. Furthermore, pathogenic PSEN mutations consistently destabilize the E-S interaction with APP- and $A\beta_n$- substrates, leading to enhanced dissociation/release of aggregation-prone, longer Aβ peptides. Similarly, several FAD-causing APP mutations destabilize the E-S complex as well, priming "*de novo* long Aβ substrates" for dissociation. These studies provide a unifying and coherent rationale for how FAD causative mutations affect γ-secretase processivity. In the context of sporadic AD, it is important that fever-like increase in temperature- or exogenous compound- induce destabilization of *wild type* E-S complexes *in vitro* and *in vivo* which is sufficient to produce amyloidogenic Aβ peptides. Our work opens new avenues for the discovery of γ-secretase stabilizing compounds (GSSC) as potential therapeutics for AD.

Results

Progressive destabilization of wild type γ-secretase-APP interactions leads to FAD-like Aβ profiles

γ-Secretase sequentially cuts APP to generate Aβ peptides of different length ($A\beta_n$) (Takami et al., 2009) in a process that involves the formation of different E-S complexes (**Figure 1A**). To investigate the relative stabilities of the consecutive E-S complexes, we performed i*n vitro* γ-secretase activity assays across a temperature gradient (from 37°C to 65°C), using purified PSEN1/Aph1A γ-secretase and the APP_{C99}-3XFLAG substrate. Increments in temperature from 37 to 55.1°C had a modest effect on the

initial endopeptidase ε-cleavage (100 ± 15 %) (**Figure 1B**), but progressively decreased enzyme processivity, as indicated by the increase in production of long Aβ peptides (**Figure 1C**). Specifically, quantitative analyses of substrates ($A\beta_{42}$ and $A\beta_{43}$) and products ($A\beta_{38}$ and $A\beta_{40}$) of the fourth γ-secretase catalytic cycle demonstrates the progressive drop in enzyme processivity (**Figure 1D**). This provides an estimate of their "optimum temperature" of production, as well as the temperature at which production drops to 50% of the initial levels at 37°C (Tm) (**Figure 1E**, dotted line). The optimum temperatures for $A\beta_{38}$ (95%CI= 31.9 to 36.9 °C), $A\beta_{40}$ (95%CI= 38.3 to 40.7 °C), $A\beta_{42}$ (95%CI= 43.9 to 46.4 °C) and $A\beta_{43}$ (95%CI= 47.8 to 49.6 °C) directly correlate with the length/hydrophobicity of the corresponding peptide, and the same conclusion can be derived from the Tm values (**Table S1 for Tm ± 95% CI**). Our data reveal that the E-S complex stability correlates with the substrate length, implying that sequential γ-secretase cleavages on APP progressively decrease E-S stabilities and thus increase the probability of E-S dissociation and Aβ release.

Next, we evaluated the temperature dependencies of the γ-secretase ε-cleavage of Notch (**Figure 1F**) (De Strooper et al., 1999). The results (**Figure 1G** vs. **Figure 1B**; Tm 95% CI: 57.4°C-59.2°C vs. 60.0°C-62.4°C, Notch and APP respectively) indicate that although distinct the stabilities of both APP and Notch (ε-) E-S complexes are higher than any of the (γ-) E-$A\beta_n$ complexes (**Table S1B).** Thus, despite strong differences in primary sequence, longer (ε)-substrates display higher stability of E-S interactions than seen with shorter (γ) substrates. The relative higher stability of the endo-proteolytic cleavage (vs. the γ-cleavages) implies that the associated physiologically relevant signaling cascades mediated by ε-cleaved intracellular protein domains, are relatively more resistant to destabilizing perturbations.

Membrane components stabilize γ-secretase-substrate interactions.

The evaluation of γ-secretase kinetics in CHAPSO solubilized conditions is a common practice in the field. However, compared to cell-based assays (Sato et al., 2003), these conditions promote the generation of long $A\beta_{\geq 42}$ peptides. Based on the experimental evidence above, we hypothesized that detergent extraction, similarly to increasing temperature, might destabilize γ-secretase and therefore impair processivity. Hence, we assessed γ-secretase function in CHAPSO resistant membranes (DRMs), an alternative and well validated cell-free system for the study of γ-secretase activity (Kakuda et al., 2012; Matsumura et al., 2014; Szaruga et al., 2015; Wahrle et al., 2002) that yields similar Aβ ratios as cell-based assays (**Figure S1B-C**). We prepared DRMs from five post-mortem human brain samples of control subjects. Thermal analysis of ε-endopeptidase activity evaluated by *de novo* AICD generation, revealed no detrimental changes over the 37-65°C temperature interval (**Figure 1H**), demonstrating the stabilizing effect of DRMs on this activity (compare **Figure 1H vs. 1B**). In contrast, a mild increment in temperature significantly affected γ-secretase processivity (73.2 % ± 10.0 at 40°C; mean of means ± SD) (**Figure 1I**) to a level similar as seen with FAD patient brain samples with further temperature increments. s **(**59.4% ± 2.3 at 45°C and 20.3% ± 5 at 55°C; mean of means ± SD of control brain DRMs vs. 44.4% ± 13.6 at 37°C mean of means ± SD of 22 FAD brain DRMs; data for FAD taken from Figure

2 in (Szaruga et al., 2015)). These studies demonstrate that the membrane environment shapes Aβ profiles by stabilizing the most labile γ-secretase–$Aβ_n$ complexes and that thermal destabilisation of wild type γ-secretase leads to Aβ profiles similar as those seen in FAD.

$Aβ_n$ substrate length is a determinant for E-S complex stability

Given that E-S complexes containing relatively long $Aβ_n$ peptides (n= 45, 43 and 42) are the most labile and therefore susceptible to dysregulation, we investigated the conversion of synthetic $Aβ_{46}$ to $Aβ_{43}$ (**Figures 2A and 2B**) and $Aβ_{45}$ to $Aβ_{42}$ (**Figures 2D and 2E**) by purified wild type enzyme at various temperatures. The substrates differ only by one amino acid but display distinct thermal susceptibilities (**Figure 2B vs 2E**), indicating that the relative stability of the E-$Aβ_{46}$ complex is (two times more stable than the E-$Aβ_{45}$ complex at 51°C, kinetic parameters summarized in **Table S2**). The importance of substrate length for E-S stability is further illustrated by the observation that generation of $Aβ_{43}$ from $Aβ_{46}$ occurs with similar efficiencies at 37°C and 51°C (**Figure 2B and Table S2**), while its further conversion (in the same reaction mix) to $Aβ_{40}$ drastically decreases (~82%) at 51°C (**Figure 2C**). Similarly, the $Aβ_{40}/Aβ_{43}$ and $Aβ_{38}/Aβ_{42}$ ratios (**Figure 2G**), which provide estimates for the efficiencies of the corresponding cleavages, indicate that γ-secretase cuts $Aβ_{43}$ more efficiently than $Aβ_{42}$ already at 37°C.

In addition, we investigated the processing of the synthetic $Aβ_{43}$ by wild type γ-secretase. This peptide was cut in a "one-turnover" process, as no $Aβ_{37}$ was generated in the assay, and its conversion to $Aβ_{40}$ occurred very inefficiently already at 37°C (**Figure S2**) (~0.2% of the efficiency of $Aβ_{45}$ to $Aβ_{42}$ at 37°C). Remarkably, the efficiency of the $Aβ_{43}$ to $Aβ_{40}$ cleavage is higher when a "de novo $Aβ_{43}$" (generated from $Aβ_{46}$) is processed than when $Aβ_{43}$ is given as initial substrate (**Figure 2G vs. S2**). This suggests that pre-established interactions between enzyme and Aβ precursor contribute to the stability of the newly generated E- $Aβ_n$ complex.

The decreasing stabilities of E-S complexes with peptides <$Aβ_{46}$ strongly point to a critical Aβ substrate length for efficient γ-cleavage processing. Remarkably, the effect of temperature on the catalytic efficiencies is explained by decreased proteolytic rates (Vmax) (**Table S2**) rather than by changes in affinity (Km) as will be discussed below. Finally, determination of the apparent substrate equilibrium dissociation constant (Kd) revealed a substantial difference between the relative affinities for APP_{C99} vs. $Aβ_{43}$, $Aβ_{40}$ and $Aβ_{38}$ peptides (**Figure S3A and Table S4**). This provides direct experimental evidence that substrate length affects the strength of E-S interactions (**Figure 2H**).

AD-linked PSEN mutations destabilize γ-secretase–substrate complexes

We next investigated the effect(s) of AD-linked PSEN mutations on the stability of the γ-secretase cleavages. We selected eight pathogenic PSEN1 mutations (P88L, Y115H, M139V, L166P, R278I, E280A, G384A and L435F) that impair the ε- endo and γ- carboxypeptidase-like activities to different extents (Chávez-Gutiérrez et al., 2012; Saito et al., 2011; Veugelen et al., 2016), differ in age of onset

and location throughout PSEN1 (**Figure 3A, 3B and Table S3**). The R278I and L435F mutations additionally affect activation of the γ-secretase pro-enzyme (PSEN auto-proteolysis) and drastically reduce levels of the active γ-secretase in cells (Saito et al., 2011; Veugelen et al., 2016). Thermo-activity assays using purified wild type or mutant γ-secretase complexes and the APP_{C99}-3XFLAG substrate reveal that PSEN mutations consistently shift Tm values for AICD-3XFLAG (**Figure 3C**), $Aβ_{38}$, $Aβ_{40}$ and $Aβ_{42}$ production (**Figure 3D; upper, middle and lower panels, respectively**). Thus, clinical mutations consistently destabilize, to different extents, the productive interaction with APP_{C99} and de novo $Aβ_n$ substrates, relative to the wild type enzyme (**Table S1**).

To investigate the underlying cause of the observed destabilization of E-S complexes by PSEN mutations we evaluated directly their effects on the intrinsic stabilities of the protease. We subjected purified wild type and mutant enzymes to increasing temperatures for 15 minutes, pelleted thermally induced aggregates by ultracentrifugation and measuredthe remaining soluble PSEN NTF and CTF fractions by immunoblot,. All tested FAD mutations increase aggregation and thus impair the intrinsic thermostability of γ-secretase (**Figure S4A, 4B**). Also the binding of conformational-sensitive Nanobodies (Nb4- and Nb28) to γ-secretase is less thermostable with mutant γ-secretases (PSEN1-L166P, E280A or G384A) than with wild type complex (Figure S4C, dotted line). The P88L, R278I and L435F γ-secretase complexes drastically reduce the endopeptidase activity and produce mostly $Aβ_{43}$ and longer peptides at 37°C (Ohki et al., 2014; Saito et al., 2011; Veugelen et al., 2016). The activities of these mutants rapidly decay with increasing temperature (**Figure 3C and Figure S5**), indicating severe destabilisation of the intermediary and initial E-S complexes. This likely explains their inefficient endo- and carboxypeptidase activities at normal body temperature. The other PSEN mutations display significant, but relatively mild destabilizing effects on $Aβ_{38}$ production (**Figure 3D; upper panel**, see also the corresponding Tm values in **Table S1**). Given that $Aβ_{42}$ is the main precursor of $Aβ_{38}$, the relatively fast decay in $Aβ_{38}$ production reflects the high probability of $Aβ_{42}$ release.. The destabilizing effects induced by pathogenic mutations become more clear when analyzing $Aβ_{40}$ and $Aβ_{42}$ generation, which reflects the processing of $Aβ_{43}$ and $Aβ_{45}$ substrates, respectively (**Figure 3D; middle and low panels**). The Tm values (**Table S1**) determined for pathogenic PSEN variants are consistently lower than the corresponding wild type values. These findings indicate that the destabilizing effects induced by PSEN mutations result in increased E-$Aβ_n$ complex dissociation rates and hence enhance release of longer Aβ peptides, therefore decreasing productive interactions. We note that increased dissociation does not necessarily imply a lower affinity for the Enzyme/Substrate interaction (Kd) as will be demonstrated below. The clinical relevance of the AD-mutant induced destabilization of E-S complexes is supported by the remarkable correlation between the age of onset for 5 out of 8 PSEN mutations and theirdestabilizing effects (Tm) on ε-endopeptidase (**Figure 3E**) or γ-carboxypeptidase activity (**Figure 3F**). Intriguingly, patients carrying the P88L, R278I and L435F mutations, have a ”delayed” age of onset despite their drastic effect on γ- processivity (generation of $Aβ_{≥43}$). Possibly the extreme low global activity of these mutant alleles (generating very small amounts of $Aβ_n$) counteracts the deleterious

effects that may be associated with the enhanced production of long Aβ peptides. This would explain the paradox that the most severe loss of function mutations result in a later age at onset than the rest

AD-linked APP mutations prime "γ-secretase-Aβ complexes" for dissociation

Our studies place the E-S complex stabilities central to FAD pathogenesis. We wondered whether pathogenic mutations in APP would similarly affect these assemblies. We tested T43I-, I45F-, V46F- and V46I-APP mutations (**Table S3**). At the endopeptidase level, three mutations exerted mild destabilizing effects while T43I did not differentiate from the wild type (**Figure 4A and Table S1**). When the E280A-PSEN mutation, which has a mild destabilizing effect (**Figure 3C**) was used, however, endo-proteolytic processing of the V46F and I45F-APP substrates demonstrated additive destabilizing effects (**Figure 4B and Table S1**), indicating converging detrimental effects of APP and PSEN clinical mutations.

We therefore studied in depth the processing of mutant APP substrates in DRMs prepared from insect cells expressing wild type γ-secretase (Acx et al., 2014). AICD and $A\beta_n$ products were directly quantified by MALDI-TOF and MALDI-FTICR mass spectrometry without any enrichment steps (**Figure S6A-C**). Employing this approach allowed us to verify the production of $A\beta_{45}$ (**Figure S6C**), the stabilizing effect of the membrane-like environment and the relatively weak effect of pathogenic mutations in APP on AICD generation (**Figure S6D vs. Figure 4A**). We also confirmed the previously observed shift in the position of the ε-cleavage that favours the $A\beta_{48}$ -> $A\beta_{42}$ product line linked to the T43I, V46F and V46I substitutions, but not to the I45F variant (Bolduc et al., 2016; Chávez-Gutiérrez et al., 2012; Dimitrov et al., 2013) (**Figure 4C**).

As shown above, E-S complexes containing $A\beta_n$ peptides are more prone to destabilization (**Figure 4D**). Beyond 58°C, increased release of the precursor $A\beta_{45}$ is seen in urea-based gel electrophoresis (**Figure 4J, 55°C**). Similar analyses revealed that the T43I substitution increases the thermal susceptibilities for the $A\beta_{38}$, $A\beta_{40}$, $A\beta_{42}$ and $A\beta_{43}$ products (**Figure 4E compare to 4D**), indicating a mutant-induced destabilizing effect on E-S complexes for long $A\beta_{\geq 43}$ substrates. In support of this, T43I-mutant Aβ profiles show long Aβ products at 37°C and their levels increase with temperature (**Figure 4J, Figure S6E**). As the T43I mutation drastically shifts the position of the ε-cleavage to favor $A\beta_{48}$ generation (**Figure 4C**), we speculate that the longer Aβ products are $A\beta_{45}$ and/or $A\beta_{48}$.

As previously reported, neither $A\beta_{40}$ nor $A\beta_{43}$ products are generated from the mutant I45F-APP substrate due to a mutant-induced product-line shift that involves the conversion of $A\beta_{46}$ to $A\beta_{42}$ (Bolduc et al., 2016). Aβ profiles confirmed the presence of $A\beta_{38}$ and $A\beta_{42}$, and showed at 37°C the presence of a longer Aβ product with $A\beta_{46}$ mobility (**Figure 4J**). Increments in temperature induced progressive decrements in $A\beta_{38}$ (**Figure 4F**) and increased $A\beta_{42}$ and $A\beta_{46}$ levels over the 37-65°C interval (**Figure 4J**).

Finally, the thermo-activity assays with V46F and V46I APP mutations show enhanced $A\beta_{38}$ and $A\beta_{42}$ decays (**Figures 4G and 4H, respectively and Figure S6F**) and increasing $A\beta_{43}$ levels at temperatures

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above 50°C (**Figures 4G and 4H, respectively and Figure S6G**), relative to the wild type substrate. Thus, these pathogenic substitutions (in particular the V46F mutant) destabilize the γ-secretase-$A\beta_{43}$ interaction and may also impair mutant E-S complexes containing Aβ peptides longer than 43 amino acids. We expressed transiently the V46F/I substrates in cells and quantified by ELISA secreted $A\beta_{40}$ and $A\beta_{43}$ peptides. As expected, the $A\beta_{40}/A\beta_{43}$ ratio was strongly reduced confirming the destabilized γ-secretase-$A\beta_{43}$ interaction (**Figures 4I**). Urea based electrophoresis demonstrates enhanced $A\beta_{45}$ production from the V46I substrate at 55°C, relative to the wild type DRM reaction (**Figure 4J**).

Overall, our data indicate that the T43I, I45F and V46I mutations destabilize the E-S complexes with $A\beta_{48}$, $A\beta_{46}$ and $A\beta_{45}$, respectively, leading to dissociation and release of these long Aβ peptides. Intriguingly, the V46F mutant destabilizes the 'wild type' γ-secretase-$A\beta_{43}$ complex. The V46F substitution drastically shifts the ε-cleavage position to favour the $A\beta_{42}$ product line (**Figure 4C**), and nevertheless still generates substantial amounts of $A\beta_{40}$ at 37°C (**Figure 4J**). This suggests that the phenylalanine substitution promotes the alternative cleavage of $A\beta_{48}$ to $A\beta_{43}$ ($A\beta_{40}$ precursor) (Matsumura et al., 2014). However, the additional effects of this mutation on docking/presentation of the substrate to the catalytic site and/or endopeptidase cleavage specificity (different AICD and therefore long Aβ products are generated, **Figure 4C**) may contribute to the observed effects. Together, our data demonstrate that APP mutations located around the γ-cleavage sites impact the stability of E-$A\beta_n$ complexes and consequently enhance product dissociation and the release of long amyloidogenic Aβ by a similar mechanism to that proposed for PSEN pathogenic mutations (**Figure 4B**).

Exogenous factors stabilize or destabilize γ-secretase-substrate interactions

Previous studies have shown that diverse compounds, referred to as γ-secretase inverse modulators, mimic FAD-linked mutations by enhancing $A\beta_{42}$ generation from the wild type enzyme (Kukar et al., 2005). In contrast, γ-secretase modulators (GSMs) (Weggen et al., 2001), including endogenous metabolites (Jung et al., 2015), enhance protease processivity (Chávez-Gutiérrez et al., 2012; Takeo et al., 2014). However, their mechanisms of action have remained elusive.

We tested three different GSM chemistries (**Figure 5A**) in γ-secretase thermo-activity assays. As expected, the direct γ-secretase modulators GSM A and B and the inverse modulator fenofibrate (GSM C) (Kukar et al., 2005) increased and decreased the conversion of $A\beta_{42}$ to $A\beta_{38}$ at 37°C, respectively (**Figure 5B**). Most notably, the addition of 10 μM direct GSM to wild type enzyme maintained elevated protease processivity ($A\beta_{38}/A\beta_{42}$) over the 37-55°C interval (**Figure 5B**), thus apparently stabilizing the γ-secretase-$A\beta_{42}$ interaction. The compound mediated stabilization is also observed to a certain extent at the endopeptidase level (AICD production), especially with GSM B (**Figure 5C**).

Remarkably, fenofibrate leads to important reductions in AICD production at increasing temperatures, which is indicative of a strong destabilizing effect on γ-secretase-APP substrate complexes. Our data provide mechanistic insights into the mode of action of therapeutically relevant compounds.

Furthermore, these results highlight the possibility that environmental factors affecting the stability of the most labile E-S complexes (containing short $A\beta_n$) could alter the risk for sporadic AD.

Elevation in body temperature modulates γ-secretase activity *in vivo*

Given the observed high thermal susceptibility of the PSEN/γ-secretase activity (**Figure 1I**), we evaluated the effect of elevated temperature in intact cells. HEK293 cells stably overexpressing human $APP695_{KM670/671NL}$ (Swedish mutation) where incubated for 1 hour at 37°C or at 42°C. Cell viability was not affected by this short incubation time. ELISA quantification of secreted Aβ showed a significant increase of total Aβ produced, especially of $A\beta_{43}$ (**Figure 6A and 6B**) and a significant reduction in the $(A\beta_{38}+A\beta_{40})/(A\beta_{42}+A\beta_{43})$ ratio (products/substrates of the 4th catalytic cycles) at 42°C, relative to 37°C (**Figure 6C**).

We also induced fever in mice carrying humanized Aβ sequence and the AD-linked Swedish (KM670/671NL) APP mutation (Saito et al., 2014) with an injection of 30µg LPS intraperitoneally and maintained in a warmed cage (~38.5°C) (Jiang et al., 1999). The treated mice reached a body temperature of 40°C (± 0.5°C) 25 minutes post-injection, which was maintained for maximally 2 hours (**Figure S7**). Control animals injected with saline solution and kept at room temperature (RT, ~23°C) had body temperatures around 38°C. Quantification of steady-state Aβ levels in plasma by ELISA revealed increased total Aβ levels (1.88 fold) in treated vs. control mice and relative increments of 1.51, 1.94 and 1.82 fold for $A\beta_{38}$, $A\beta_{40}$ and $A\beta_{42}$, respectively (**Figure 6D**). Accordingly, the $A\beta_{38}/A\beta_{42}$ ratio is lowered in the treated mice (**Figure 6E**). Other factors than elevated body temperature might have contributed to the observed changes in Aβ; however, the results align with the cell culture experiments. Thus elevated body temperature augments γ-secretase activity and impairs the efficiency of the 4th γ-cleavage.

Discussion

In this study we have shown that clinical mutations in PSEN and in APP destabilize primarily the intermediary E-S complexes involved in the sequential processing of APP by γ-secretase, leading to enhanced E-S dissociation and thereby release of longer, more amyloidogenic Aβ peptides. We used progressive thermal destabilization of the wild type enzyme to evaluate the strength of the different E-S interactions (**Figures 1A and 1E**), and demonstrated that increments in temperature mimic the effects of clinical AD mutations. Our studies reveal a direct correlation between substrate length and E-S complex stability, indicating that each γ-secretase cleavage reduces the stability of the subsequent E-S complex and thereby progressively shifts the equilibrium towards dissociation and Aβ release. Direct determination of the Michaelis constants for $APP\text{-}C_{99}$ (Km ~ 400-800 nM (Chávez-Gutiérrez et al.,

2012; Funamoto et al., 2013)) and for $A\beta_{46}$, $A\beta_{45}$ or $A\beta_{43}$ substrates (Km ~3 µM, **Table S2**) as well as relative affinities for APP-C_{99}, $A\beta_{43}$, $A\beta_{40}$ and $A\beta_{38}$ (**Figure S3A)** support this concept.

We propose that γ-secretase-APP_{C99} complexes are stabilized by a network of weak bonding interactions along the substrate transmembrane domain (TMD) (global fitting). In this scenario the stabilizing binding energy depends critically on the area of interacting surface, i.e. on the substrate length, while the side chains of the constituting residues are not the main driving force for the formation nor for the stabilities of the E-S complexes. This view is supported by the known relaxed γ-secretase substrate specificity. However, in sharp contrast, we observed that single amino acid substitutions in APP can exert a profound destabilizing effect on E-$A\beta_{n,}$ complexes.. These findings imply that the type of interactions involved in the stabilization of the initial ε-E-S vs. the consecutive γ- E-S complexes are fundamentally different.

While the nature of the E-S interaction remains unknown, the recent structure of γ-secretase in complex with a co-purifying type I transmembrane protein provides interesting insights. As shown in **Figure 7**, a short helical structure anchors the putative substrate to PSEN-NTF, while an unstructured stretch of ~5 amino acids extends through a wide channel to reach the catalytic residues (Bai et al., 2015b). In our view, this co-structure could illustrate how a *de novo* $A\beta_n$ product interacts with PSEN before it engages in the next catalytic cycle, or is released. In the former case, further unwinding of the N-terminal helix must occur in order to fill the S1'-S3' enzyme pockets (Bolduc et al., 2016) during catalysis(**Figure 7**). This will lead to further destabilization of the anchor helix, which weakens the E-S assembly and importantly, confers progressively more significance to other (side chain dependent) interactions along the $A\beta_n$ substrate. This model provides an explanation for why AD-linked substitutions in APP ($A\beta_{42-46}$) destabilize γ-secretase-Aβ but not γ-secretase-APP_{C99} complexes. The presented data indicate that $A\beta_{45}$ and $A\beta_{46}$ substrates are of a critical length as the following γ-cut drastically reduces the stability of the subsequent γ-secretase-$A\beta_{43/42}$ complexes (**Figures 3C and 3F**). According to the E-Aβ interaction model (**Figure 7**), this can best be understood by taking into account the fact that unstructured C-terminal stretches of ~5 amino acids leave N-terminal helical anchors of 13 aa, 12 aa, 10 aa or 9 aa in the $A\beta_{46}$, $A\beta_{45}$, $A\beta_{43}$ or $A\beta_{42}$ peptides, respectively. It appears that an N-terminal helical anchor with at least 3 turns ($A\beta_{46}$) stabilizes E-S interactions and promotes efficient γ- processing.

Pathogenic PSEN1 mutants, irrespective of their nature or position, destabilize E-S complexes (**Figure 3)** and previous work analysing the ε-cleavage show that their effects are not explained by lower substrate affinities, but rather by reduced catalytic rates (decreased Vmax, see also **Suppl. Table 2**). Similarly, elevated temperature lowers Aβ processing efficiencies by reducing proteolytic rates. This indicates that thermal or mutant- induced destabilization does not affect directly the formation of 'γ-secretase-$A\beta_n$' complexes but instead disturbs a subsequent step in catalysis. In agreement, FAD-linked mutants in PSEN do not directly affect APP_{C99} nor $A\beta_n$ substrate binding to γ-secretase (**Figure S3C-**

D). Furthermore, the stabilizing GSMB compound does not change APP_{C99} nor $A\beta_{43}$ binding to the wild type complex (**Figure S3B**). Thus, destabilization of the productive E-S interactions is not due to alterations in the initial formation of 'γ-secretase-APP' complexes, but a consequence of thermal/mutant/compound- induced disturbances during the subsequent catalysis.

Upon E-S formation, the complex can either decompose back to free enzyme and substrate or undergo catalysis via a transition state intermediate (E-S* complex), which involves structural rearrangements in both the enzyme and the substrate. The γ-secretase proteolytic mechanism likely involves, as explained, local helix unwinding prior to cleavage and the resultant backbone break must have a destabilizing effect on the remaining helical structure of the subsequent *de novo* $A\beta_n$ substrates (**Figure 7**).

Although our knowledge of intramembrane proteolytic mechanisms remains very limited, insights from the rhomboids are already very apparent. For instance, the substrate (TMD) helical propensity is likely pivotal to the E-S interaction and proteolysis is driven by reaction kinetics, rather than substrate affinity (Dickey et al., 2013; Moin and Urban, 2012). We propose that FAD-linked PSEN mutations, or increased temperature, disturb the stabilization of the E-S* transition state, promoting non-productive dissociation (E-S*→ E+S) or restoration of the E-S state (E-S ← E-S*). In support FAD-linked G384A and Delta Exon 9 PSEN mutations decrease the affinity for the transition-state analogue L-685,458 (Svedruzic et al., 2012). In the case of the APP-CTF substrate, which remains anchored to the membrane, a destabilizing effect implies the re-initiation of the proteolytic process (E-S formation). The situation is fundamentally different in the case of $A\beta_n$ substrates, as they are no longer anchored in the membrane and their release and dilution into the extracellular milieu makes re-association with the enzyme unlikely. In support of our model, others have shown that PSEN pathogenic substitutions increase the dissociation rate of the γ-secretase-$A\beta_{42}$ complex (Okochi et al., 2013).

It is highly relevant that both APP and PSEN mutants promote the release of relatively long $A\beta_{\geq 43}$ peptides. Thus, while increments in toxic $A\beta_{42}$ are proposed as drivers of FAD (Potter et al., 2013), our findings raise the possibility that even longer Aβ species may have high pathogenic relevance.

These studies help to understand the mechanism of action of a major class of drug candidate for AD therapeutics targeting γ-secretase, i.e. the GSMs (**Figure 5**). The insights are also relevant to explain the inverse correlation between generation of long $A\beta_{42/43}$ peptides and membrane thickness (Holmes et al., 2012; Winkler et al., 2012) and the high sensitivity of γ-secretase function to lipid environment changes (Holmes et al., 2012; Osenkowski et al., 2008).

Besides providing fundamental novel insights into the working of γ-secretase, the current report raises some additional interesting pathophysiological considerations. The significant changes in γ-secretase processivity observed upon mild temperature increments *in vitro* and *in vivo* suggest that fever could promote amyloidogenic Aβ generation. While further work is needed to investigate the relationship

between fever and the occurrence of AD, it is interesting to notice that carriers of the M694V pyrin mutation associated with Familial Mediterranean Fever have a 3-fold higher risk for sporadic *early-onset* AD than those with normal alleles (Arra et al., 2006). It is not clarified yet whether this is caused by the fever or by other (unknown) effects of this mutation.

In conclusion, our findings reveal substantial insights into the structural and kinetic γ-secretase mechanisms and *support a unifying model for AD causative mutations* that places generation of longer Aβ peptides central in AD pathogenesis. Furthermore, these studies provide a novel conceptual framework for investigating γ-secretase (dys)function in sporadic AD, as the demonstrated fragility of γ-secretase processivity suggests that mechanisms similar to those underlying FAD may increase the risk of sporadic AD in a subgroup of patients (Szaruga et al., 2015). Finally, our findings may guide novel efforts to develop safe therapies that target γ-secretase, i.e. the generation of γ-secretase (E-S) stabilizing compounds (GSSC) for the prevention and treatment of AD.

Contributions

M.S. performed experiments and analyzed the data. B.M. and C.H. performed Mass-Spectrometry data collection and processing. S.L. performed cloning, protein purification and assisted with experiments. S.V. carried out the substrate affinity determinations and nanobody work. K.H. provided technical assistance with animal work. M.M. provided Aβ ELISA antibodies. T.S. provided APP NL mice. N.C.F. and N.S.R. gave clinical input. T.D.V. and S.N.S. provided input and expertise for the determination of substrate-binding affinities. R.G., J.S. and F. R. provided input and expertise in the assessment of γ-secretase stabilities. L.C.G. designed the study and analyzed the data. L.C.G. and B.D.S supervised the research and wrote the manuscript.

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Figure legends

Figure 1. Temperature increment induces production of "pathogenic-like" Aβ profiles by wild type γ-secretase

A) Schematic representation of the Enzyme-Substrate (E-S) complexes characterizing the processing of APP by γ-secretase. **B-E)** *In vitro* activity assays with purified wild type human γ-secretase

(PSEN1/APH1a) and APP_{C99}-3xFLAG over a temperature gradient. **B)** Total AICD-3xFLAG product levels analyzed by quantitative western immunoblot (top panel) reveal similar AICD product levels (100 ± 15%) in temperatures ranging from 37 to 55.1°C (white background lower panel). Gaussian fitting indicates an optimal AICD production temperature of 44.8°C ± 0.6°C for AICD, (mean ± SE, n>5). **C)** Aβ profiles resolved in urea gels show enhanced generation of long Aβ peptides in the 37 to 60°C temperature range. Loading order: synthetic Aβ peptides ($Aβ_{1-38}$, $Aβ_{1-40}$, $Aβ_{1-42}$, $Aβ_{1-45}$ and $Aβ_{1-46}$ peptides at 1/1/1/0.1/1 molar ratios), purified APP_{C99} substrate and proteolytic reactions incubated at indicated temperatures. (*) Indicates a non-product band, present in the purified APP substrate. **D)** $Aβ_{38}$, $Aβ_{40}$, $Aβ_{42}$ and $Aβ_{43}$ product levels were quantified by ELISA. Enzyme processivity estimated by the $Aβ_{40/43}$ and $Aβ_{38/42}$ ratios (substrate/product of the 4th turnover) reveal progressive reductions in the corresponding catalytic efficiencies over the 37°C - 51°C range. **E)** Gaussian fit on temperature-induced decays of $Aβ_{38}$, $Aβ_{40}$, $Aβ_{42}$ and $Aβ_{43}$ products reveal a correlation between the optimal temperature of production (interpolated, vertical lines) and peptide length (maximal interpolated Aβ levels adjusted to 100%; mean ± SEM, n=4). **F)** γ-Secretase endopeptidase (ε) and carboxypeptidase-like (γ) cleavage sites on transmembrane domains of APP (Takami et al., 2009) or Notch1 substrates (Okochi et al., 2006) (black and grey arrows on APP describe the two production pathways shown in figure 1A). **G)** Thermo-activity assays with purified wild type γ-secretase show significant differences in thermal susceptibilities of the ε-cleavages of Notch-3xFLAG (data in red) (mean ± SD, n=3) vs. APP_{C99}-3xFLAG (data in gray, Figure 1B, $P_{value}< 0.01$). Dotted lines through the manuscript indicate the temperatures at which production reaches 50% of their initial levels at 37°C (Tm). **H-I)** In vitro activity assays using DRMs prepared from postmortem human brain samples and APP_{C99}-3xFLAG as substrate were incubated over a temperature gradient. **H)** D*e novo* AICD levels determined by quantitative western blot (top panel) show no significant changes over the 37°C-55°C temperature interval (mean of means ± SD, 5 patient samples, n ≥2). **I)** ELISA quantified $Aβ_{38}$ and $Aβ_{42}$ products demonstrate that increments in temperature lead to progressive impairment in γ-processivity, specifically at the 4th catalytic turnover; (mean of means ± SD, 4 patient samples, n=2, One-way ANOVA and Dunnett's post-test P<0.0001). Previously published $Aβ_{38/42}$ ratios (Szaruga et al., 2015) were determined at 37°C for DRMs prepared from post-mortem brain samples of 22 FAD patients carrying 9 different PSEN mutations (grey area, mean of means ± SD).

Figure 2. Kinetic analyses of the sequential processing of $Aβ_{46}$ and $Aβ_{45}$ by wild type γ-secretase

A, D) Schematics for γ-secretase processing of $Aβ_{46}$ and $Aβ_{45}$. **B-G)** Thermo-activity assays using purified γ-secretase and synthetic $Aβ_{46}$ or $Aβ_{45}$ as substrates. **B)** $Aβ_{43}$ product levels at 37° and 51°C was fit with a Michaelis-Menten model (fit ±95% CI), (mean ± SEM , n=4). Notice that part of the *de novo* $Aβ_{43}$ generated in the reactions is further processed to $Aβ_{40}$ or $Aβ_{38}$; thus total $Aβ_{43}$ generated is estimated here as $Aβ_{43}$+$Aβ_{40}$+$Aβ_{38}$. **C)** Subsequent conversion of $Aβ_{43}$ into shorter $Aβ_{40}$ (or $Aβ_{38}$, not shown) at 37°C or 51°C; (mean ± SEM, n=4). **E)** Processing of $Aβ_{45}$ into $Aβ_{42}$ fit with a Michaelis-

Menten model (fit ±95% CI) and **F)** subsequent cut to $A\beta_{38}$ at the indicated temperatures (mean ± SEM, n=3). Notice that part of the *de novo* $A\beta_{42}$ generated in the reactions is further processed to $A\beta_{38}$; thus total $A\beta_{42}$ is calculated as $A\beta_{42}+A\beta_{38}$. **G**) $A\beta_{40}/A\beta_{43}$ and $A\beta_{38}/A\beta_{42}$ ratios indicate that $A\beta_{43}$ is less efficiently processed than $A\beta_{42}$ at 37°C; while both cleavages are strongly impaired at 51°C. Graph includes all data points shown in panels C and F (mean ± SEM, t-test, $P_{value}< 0.0001$). **H)** Sequential γ-secretase cuts on APP progressively decrease E-S complex stability and increase the probability of dissociation.

Figure 3. AD-linked PSEN1 mutations impair the stability of γ-secretase-substrate complex

A) Scattered distribution of PSEN residues mutated (red) in FAD. **B)** 3-D Location of the selected PSEN1 mutations (red), except for the R278 which is not resolved in the 3D structure. Catalytic Asp residues shown in yellow. **B1)** lateral and **B2)** bottom views of PSEN1 structure (brown) with a co-purifying peptide (grey) (PDB: 5fn2). **C-D)** Thermo-activity assays using purified wild type or mutant γ-secretase complexes and C99-3xFLAG substrate incubated for 20 min at the indicated temperatures, except for the severe P88L mutant which activity was measured after 1 h and its concentration was 10x higher. **C)** Representative immuno-blots showing AICD-3XFLAG levels generated by the different protease complexes (top panel). Lower panel shows Gaussian fittings on AICD-3XFLAG product levels; (mean ± SEM, n=4) (See Table S1A for Tm ± 95%CI). Null hypothesis (one curve for all data sets) rejected; different curves for each data set with P <0.0001. **D)** Gaussian fittings on ELISA quantified $A\beta_{38}$, $A\beta_{40}$ and $A\beta_{42}$ peptides produced by wild type or mutant γ-secretase complexes at the indicated temperatures (top, middle and lower panels, respectively); (mean ± SEM, n=4). The relative shifts in apparent Tm's (dotted line) demonstrate the mutant induced destabilizing effects. Different curves for each data set in $A\beta_{42}$ panel (lower) with P <0.0001. **D-E)** Apparent Tm ± 95% CI for AICD generation and $A\beta_{42}$ production by mutant enzymes vs. the corresponding age of onset of AD in patients (AICD: y = 0.3678*x+ 38.66; ± 95% CI for 5 out of 8 mutants and $A\beta_{42}$: y = 0.3509*x + 32.48; ± 95% CI). Notice that P88L, L435F and R278I are the more severe 'loss' of function mutations, and apparently show a delayed age of onset (y = 0.8843*x + 7.971, dotted line) (see discussion).

Figure 4. AD-linked APP mutants consistently affect the stability of γ-E-S complexes

AICD product levels generated in thermo-activity assays from the indicated mutant APP_{C99}-3XFLAG substrates and purified **A)** wild type or **B)** E280A-PSEN1 mutant γ-secretase complexes. Gaussian fittings on *de novo* AICD-3XFLAG levels (mean ± SEM, n≥3). Different curves for each data set with P <0.0001 in (B). (See Table S1A for 95% CI). **C-H)** MALDI-TOF Mass spectrometry analysis of thermo-activity assays using DRMs associated wild type γ-secretase complex and purified wild type or mutant APP_{C99}-3xFLAG substrates. **C)** *De novo* AICD products at 37°C; (mean ± SEM, n≥4, except for I45F mean ± SD, n=2) and **D-H)** Aβ products generated from the indicated APP substrate over the indicated temperature gradient. Aβ product levels are normalized to total endopeptidase activity (total

AICD levels); (mean ± SEM, n≥4, except for I45F mean ± SD, n=2). **I)** $A\beta_{40}/A\beta_{43}$ ratios determined by ELISA in the extracellular media of HEK293 cells transiently expressing wild type or mutant APP substrates (n=4, one-way ANOVA and Dunnett's post-test, *** P≤ 0.001). **J)** Aβ profiles generated at 37°C and 55°C resolved in urea-based gels. Synthetic $A\beta_{1-38}$, $A\beta_{1-40}$, $A\beta_{1-42}$, $A\beta_{1-45}$ and $A\beta_{1-46}$ peptides mixed at 1/1/1/0.1/1 molar ratios were loaded as reference. (*) Indicates a non-product band, present in the purified APP substrate.

Figure 5. Evaluation of modulators in γ-secretase thermo-activity assays

A) γ-Secretase modulators used in the study. **B-C)** In vitro thermo-activity assays with purified wild type γ-secretase and wild type C99-3xFLAG in presence of direct (GSM(A), GSM(B)) or inverse GSM C (Fenofibrate). **B)** Direct modulators enhance γ-cleavage efficiency over temperatures ranging from 37 to 55°C, relative to DMSO control, (mean ± SEM, n=4). Notice the 2x and 8x increase of the 4^{th} cycle with GSM A and GSM B respectively at 37°C. **C)** The stabilizing/destabilizing effects of GSMs are also observed at the first endoproteolytic cleavage of C99. The graph shows Gaussian fitting on AICD product levels, (mean ± SEM, n=4; *, ** and **** indicate $P_{values}\leq$ 0.05, 0.01, and 0.0001, respectively).

Figure 6. Elevation in body temperature to fever range modulates γ-secretase activity on **A-C)** cultured cells and **D-E)** *in vivo.* **A)** ELISA quantified Aβ peptides secreted by HEK/Swe APP at 37°C and 42°C; **B)** Secreted Aβ ($\sum A\beta_{38}+A\beta_{40}+A\beta_{42}+A\beta_{43}$) and **C)** $(A\beta_{38} + A\beta_{40}) / (A\beta_{42} + A\beta_{43})$ (products / substrates of the 4^{th} turnovers) ratio demonstrate increased Aβ secretion but decreased processivity at 42°C, relative to 37°C; (Unpaired t-tests, *p≤ 0.05). **D-E)** Fever was induced in APP NL female mice by intraperitoneal injection of 30 µg of LPS and housing in a pre-warmed cage (see **Figure S4**). **D--E)** Tukey box-and-whiskers plots for **D)** ELISA quantified Aβ steady-state levels in plasma and **E)** the $A\beta_{38/42}$ ratio show increased secreted $\sum A\beta$ ($A\beta_{38}+A\beta_{40}+A\beta_{42}$) levels and a significant reduction in protease efficiency (4^{th} catalytic turnover) in the fever group after 100 min fever period, respectively. 10 control and 10 treated animals were tested, Unpaired two tailed t-tests, *, **, *** and **** indicate $P_{values}\leq$ 0.05, 0.01, 0.001 and 0.0001, respectively.

Figure 7. Model for E-S interactions during the multiple turnover processing of APP by γ-secretase

PSEN1 structure (Lateral view, brown) (PDB: 5fn2, (Bai et al., 2015b)) with the structure of APP_{C99} (purple) (PDB:2LP1, (Barrett et al., 2012)) manually docked in the putative substrate binding (see Figure S1 A4). **A)** C99 or **C)** $A\beta_n$ interacts with PSEN before it engages in the next catalytic cycle (E-S*) or is released (E+S) (**B-D**). Unwinding of the N-terminal transmembrane helix (**B, D**) occurs in order to fill the S1'-S3' enzyme pockets (Bolduc et al., 2016) during catalysis. The progressive shortening of the N-terminal anchor progressively destabilizes γ-secretase-$A\beta_n$ complexes shifting the equilibrium towards dissociation ($A\beta_n$ release).

Supplementary Figure legends

Figure S1. A1) 3D structure of the wild type human γ-secretase (PSEN1/APH1a) in complex with the putative substrate (PDB: 5fn2, (Bai et al., 2015b)). NCT in green, APH1 in yellow, PEN2 in brown, PSEN in beige and the putative substrate peptide in red. **A2)** lateral and **A3)** bottom views of the membrane core and **A4)** manual docking of the APP_{C99} substrate (purple) (2LP1, (Barrett et al., 2012)) into the putative substrate binding pocket of the γ-secretase complex. **B-C)** DRMs prepared from human control brain samples tested in *in vitro* activity assays using 1.5μM purified wild type APP_{C99}-3xFLAG substrate. ELISA quantifications and analysis of Aβ product profiles in urea-based gels show enhanced γ-secretase processivity (relative to detergent solubilized conditions (Figure 1C, 37°C)). $Aβ_{40}$ is the main product, similar to profiles generated in cell-based assays. The results support a stabilizing effect of the membrane environment.

Figure S2. Kinetic analyses of the processing of $Aβ_{43}$ by γ-secretase. A) Schematic processing of $Aβ_{43}$ to $Aβ_{40}$ by wild type γ-secretase. **B)** ELISA quantifications of *de novo* $Aβ_{40}$ and $Aβ_{37}$ generated indicate no further processing of $Aβ_{40}$ to $Aβ_{37}$. Graph shows $Aβ_{40}$ product levels generated at 37°C fit with a Michaelis-Menten model (mean ± SEM , n=4).

Figure S3. Competition experiments to determine apparent Kd equilibrium affinity values for APP_{C99} and synthetic $Aβ_{43}$, $Aβ_{40}$ and $Aβ_{38}$ peptides. AlphaScreen bead-based proximity assay was used to detect the interaction between biotinylated purified WT (**A-D**) or FAD-linked mutant (**C and D**) γ-secretase complexes (~1 nM) and purified APP_{C99}-3XFLAG substrate (20nM) in the presence of 10 μM γ-secretase inhibitor X (Calbiochem) (Control), while competing with increasing concentrations of untagged C99 substrate (**A and C**) or synthetic Aβ peptides (**A and D). A, C and D show** average ± SE, n ≥6. Addition of 10μM GSMB does not change E- APP_{C99} nor E- $Aβ_{43}$ binding affinities (**B**, average ± SD, n=3). Data was fit to Y=(Top-Bottom)/(1+10^(X-LogIC50)) + Bottom. Note that selected FAD-linked PSEN variants (L166P, E280A and G384A) display significantly different destabilization effects on E-S complexes in our kinetic experiments (**Figure 3 C-F**).

Figure S4. Intrinsic stabilities of WT and FAD-mutant γ-secretase complexes. A-B) WT and AD-linked protease complexes were heated to 37, 45, 55 or 65°C for 15 min and immediately ultracentrifuged to pellet thermally-induced aggregates. The remaining soluble fraction was subjected to semi-quantitative SDS-PAGE/western blot and assessed for the levels of PSEN1 NTF and PSEN1 CTF. **A)** Quantification of the relative soluble amounts of WT vs AD-linked PSEN1 N-terminal and C-terminal fragments at the distinct temperatures were used to infer the intrinsic PSEN thermal stabilities (n≥6, average ± SE) and **B)** reveal significant destabilization of the tested pathogenic PSEN1 mutants at 55°C when compared to WT protease (Statistical significance: Anova and Dunnett's post-test, *P≤0.1,

P≤0.01, ***P≤0.001). **C) Biotinylated γ-secretase - His-tagged Nb interaction was measured using a bead-proximity assay (AlphaScreen). 10 nM biotinylated γ-secretase complexes were pre-incubated with 10 nM purified His-tagged nanobody (Nb)4 or Nb28 in the presence of donor and acceptor beads for 45 minutes at 30°C. The mix was then subjected to different temperatures over a gradient from 37 to 70°C for 1h. γ-Secretase-Nb mixes were all brought back to 25°C prior to Alpha-Screen binding detection. Right panel (Nb4) and left panel (Nb 28) show the progressive destabilization of Nb-γ-secretase complex for wild type and 3 selected FAD mutants (PSEN L166P, E280A and G384A) over temperature. A significant shift in the temperature at which the γ-secretase–Nb interaction drops to 50% of its initial levels is observed (**Figure S4C, dotted line**), indicating that mutant protease complexes have relatively lower thermostabilities than the wild type (for both Nb-analyses n≥5, average ± SE, Temperature vs. normalized signal data was fit to Y=100/(1+(X^HillSlope)/(IC50^HillSlope)), different curves for each data set with P<0.0001.

Figure S5. A) Activity assays performed with purified γ-secretase complexes, containing P88L-, R278I- or L435F- PSEN1 pathogenic variants over a temperature gradient. Urea–based gel electrophoresis confirms production of only long Aβ peptides (≥$A\beta_{43}$) from these protease complexes at 37°C (Ohki et al., 2014; Saito et al., 2011; Veugelen et al., 2016).

Figure S6. MS-based MALDI mass spectrometry allows detection (and relative quantification) of the two (or more) alternative ε-cleavage products ($AICD_{50}$ and $AICD_{49}$) as well as the $A\beta_{38}$, $A\beta_{40}$, $A\beta_{42}$, $A\beta_{43}$ and $A\beta_{45}$ peptides, substrates and products of the 3rd and 4th γ-secretase turnovers in both amyloid product lines. **A-B)** Illustrates the linear mode (low resolution) MALDI-TOF MS analysis of the AICD and Aβ peptide products generated by DRM-associated wild type γ-secretase from purified wild type APP_{C99}-3XFLAG in 20 min, at 37°C (upper panel) or 60°C (lower panel) in the presence or absence of the active site inhibitor X (Inh X). "No C99" denotes a no-substrate control reaction. **C)** Illustrates high resolution MALDI FT-ICR mass spectra of wild type $A\beta_{1\text{-}45}$ originating from APP_{C99}-3XFLAG in 20 min at 37°C or 60°C. The high resolution analysis allows the monoisotopic separation of the target peptide with a mass accuracy of ~1ppm calculated from the base peak (most intensive isotope, marked with a star *). "No C99" denotes a no-substrate control reaction. Please note that relative reductions in $A\beta_{38}$, $A\beta_{40}$ and $A\beta_{42}$ product levels are accompanied by increases in $A\beta_{43}$ and $A\beta_{45}$. **E)** MALDI-TOF MS analysis of the AICD products generated by (insect cells-derived) DRM-associated wild type human γ-secretase from either purified wild type or mutant APP_{C99}-3XFLAG at different temperatures. Note the increased temperatures compared to the experiments performed with detergent-solubilized enzyme (**Figures 1B and 4A**). Graphs show mean ± SEM, n=4. **D)** DRMs prepared from insect cells expressing the wild type human γ-secretase were used as source of enzyme and tested in *in vitro* thermo-activity assays using purified mutant T43I APP_{C99}-3xFLAG substrate. Aβ profiles resolved in urea gels show enhanced generation of long Aβ peptides in the 37 to 60°C temperature range. Loading order: synthetic Aβ peptides ($A\beta_{1\text{-}38}$, $A\beta_{1\text{-}40}$, $A\beta_{1\text{-}42}$, $A\beta_{1\text{-}45}$ and $A\beta_{1\text{-}46}$ peptides at 1/1/1/0.1/1 molar ratios), purified T43I

APP_{C99}-3xFLAG substrate and proteolytic reactions incubated at indicated temperatures. **F-G)** Aβ product signal intensities extracted from low resolution MALDI TOF data, generated from wild type or mutant APP substrates at the indicated temperature and normalized to total endopeptidase activity (total AICD levels); mean ± SEM, n=4.

Figure S7. Elevation in body temperature to fever range modulates γ-secretase activity *in vivo*. **A)** Fever was induced in APP NL female mice by intraperitoneal injection of 30 µg of LPS (syringe) and housing in a pre-warmed cage for 100 min. Control mice were kept at room temperature (RT, 22-24°C) and subjected to the same handling. Mice body temperature was monitored by rectal measurements every 25 min. At the end of the experiment mice were sacrificed in CO2; mean of means ± SD from 10 control and 10 treated animals.

STAR Methods

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Lucía Chávez Gutiérrez (Lucia.ChavezGutierrez@cme.vib-kuleuven.be).

Antibodies and reagents

Antibodies were purchased as follows: anti-FLAG M2 from Sigma, Alexa 790 Goat anti-Mouse IgG from Invitrogen, 82E1 against human Aβ (N-term) from IBL, biotinylated anti-mouse IgG from Vector Laboratories and streptavidin-HRP from GE Healthcare. ELISA antibodies and γ-secretase modulators were obtained through collaboration with Janssen Pharmaceutica NV, Beerse, Belgium: JRF AB038 for Aβ1-38, JRF/cAb40/28 for Aβ1-40, JRF/cAb42/26 for Aβ1-42 and detection antibody JRF/AbN/25 against the N-terminus of Aβ. Elisa kit against amyloid β (1-43) (FL) was purchased from IBL, AlphaLISA Detection Kit against amyloid β (1-40) was purchased from PerkinElmer. Fenofibrate was purchased from Sigma Aldrich. Synthetic β-Amyloid 1-38, 1-40, 1-42, 1-43 and 1-46 were purchased from rPeptide and 1-45 from Anaspec. β-Amyloid 1-43 used as standards in ELISA was from IBL and 1-40 standard in AlphaLISA from PerkinElmer. γ-Secretase inhibitor X purchased from Calbiochem. Complete protease inhibitor tablets were purchased from Sigma-Aldrich. All reagents for MALDI-Mass Spectrometry used in this study were of HLPC grade. Acetonitrile (ACN) and trifluoroacetic acid (TFA), were purchased from Merck (Darmstadt, GER). Milli-Q water (ddH_2O; Millipore) was prepared in-house. Sinapinic acid (SA; Cat. No.201345) and MALDI-MS protein calibration standard I (Cat. No.206355) were purchased from Bruker Daltonics (Bremen, Germany).

Expression and purification of wild type APP_{C99}, wild type and mutant APP_{C99}-3xFLAG and wild type Notch-based 3xFLAG substrates

Human wild type and mutant (T43I (T714I), I45F (I716F), V46F (V717F) and V46I (V717I)) APP_{C99}-3xFLAG and Notch-based (Notch-3xFLAG) substrates were expressed in COS1 or HEK293 cells. Briefly, cells were transiently transfected with pSG5-APP_{C99}-3xFLAG (wild type or mutant) or pSG5-Notch-3xFLAG vector. Before collection, cells expressing C99-3xFLAG were treated overnight with 10 µM GM GM6001 inhibitor (Sigma) to prevent its conversion to C83-3xFLAG. Cells were harvested and resuspended in 50mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1X PI and incubated on ice for 1 h. Membrane-solubilized protein fractions were obtained by ultracentrifugation at ~200000g for 20 min. Wild type APP_{C99} substrate with a PreScission Protease cleavage site and 3X-FLAG-tag (APP_{C99}–PPS-3X-FLAG) at the C-terminus was cloned in the pAcAB4 vector and expressed in Hi5 insect cells. Cells were collected 48-72 h post infection and resuspended in 50mM Tris-HCl pH 7.6, 150 mM NaCl, 1X PI. Total membranes were prepared and rinsed twice in 1x TBS, 1M NaCl and complete protease inhibitor mixture. Membranes were solubilized ON in 1x TBS, 1% n-dodecyl β-d-maltopyranoside (DDM; Anatrace) and 1X PI at 4°C. Membrane-solubilized protein fractions were obtained by ultracentrifugation at ~200000g for 45 min.

FLAG-tagged recombinant substrates were purified by immunoaffinity chromatography using the anti-FLAG M2-agarose beads (Sigma), according to the manufacturer's protocols. All substrates, except for the APP_{C99}–PPS-3X-FLAG, were eluted in 100mM glycine HCl, pH 2.7, 0.25% n-dodecyl β-D-maltoside (Sigma) and immediately neutralized to pH 7 by the addition of Tris-HCl, pH 8.0. Digestion with GST-tagged PreScission protease eluted APP_{C99}–PPS-3X-FLAG in 25mM Pipes, 150mM NaCl, 0,02% DDM, 0,5mM EDTA and 1mM DTT. Removal of the PreScission protease was done by immunoaffinity pulldown using Glutathione Sepharose 4B (GE Healthcare). Purity was assessed by SDS-PAGE and Coomassie staining (InstantBlue Protein Stain, Expedeon).

Expression and purification of γ-secretase complexes

Human wild type or mutant (P88L, Y115H, M139V, L166P, R278I, E280A, G384A, L435F) PSEN1, NCT-GFP, APH1AL and PEN2 cDNAs were cloned into the pAcAB4 transfer vector (BD Biosciences). Co-transfection of the transfer vector (containing the heterologous genes) and flashBacGold™ DNA (Oxford Expression Technologies) in Sf9 cells allowed homologous recombination and production of baculoviruses bearing the four essential subunits of the γ-secretase complex. A PreScission Protease cleaving site (LeuGluValLeuPheGln/GlyPro) and GFP were cloned at the C-terminal site of NCT. Protease complexes were expressed in Hi5 insect cells. Infected Hi5 cells were collected at 72 h post infection and lysed in 2% CHAPSO (Anatrace) buffer (25 mM Pipes pH 7.4, 300 mM NaCl, 5% Glycerol, 1X Protease inhibitors (PI). Affinity purification was carried out using a high affinity anti-GFP nanobody covalently coupled to

agarose beads (NHS-activated beads, GE Healthcare) in a 3:1 ratio (mg:ml). PreScission protease cleavage between NCT and GFP eluted untagged γ-secretase complexes (buffer composition: 25 mM Pipes pH 7.4, 150 mM NaCl, 0.5% CHAPSO, 5% Glycerol). Finally, removal of the GST-tagged PreScission protease by immunoaffinity pulldown using Glutathione Sepharose 4B (GE Healthcare) was performed and the purity of γ-secretase complexes was assessed by SDS-PAGE and Coomassie staining (InstantBlue Protein Stain, Expedeon).

Subjects

Human cortical specimens were obtained throughout collaboration with Queen Square Brain Bank for Neurological Disorders at University College London. All of the samples came from brains that were removed and placed in -80°C within 42 hours postmortem. Samples were collected following protocols approved by respective ethical boards and written legal consents for the use of organs for medical research are available for each patient. All human protocols were approved by Medical Ethics Committee UZ KU Leuven, Belgium.

Detergent resistant membrane preparation from insect cells and human brain samples

CHAPSO detergent resistant membranes (DRMs) were prepared from Hi5 insect cells overexpressing PSEN1/APH1A γ-secretase complexes (WT or mutant complexes containing PSEN1 pathogenic mutations P88L, Y115H, M139V, L166P, R278I, E280A, G384A or L435F) or human brain frontal cortices (after careful removal of leptomeninges and blood vessels). In case of human brain samples, around 200 mg blocks of tissue were homogenized in ~ 10 volumes of 10% sucrose in MBS buffer (25mM MES, pH 6.5, 150mM NaCl) containing 1% CHAPSO (Sigma) and 1X PI. DRMs from insect cells were prepared from total membranes (200 ml Hi5 cell culture). Membrane pellets were homogenized in ~ 2.5 ml of 10% sucrose in MBS buffer containing 1% CHAPSO (Sigma) and protease inhibitors. Brain or cell membrane homogenates were mixed with equal volume of 70% sucrose in MBS buffer, 4 ml was placed at the bottom of an ultracentrifuge tube (Beckman, 344059) and successively overlaid with 4 ml of 35% sucrose (MBS) and 4 ml of 5% sucrose (MBS). Samples were centrifuged at 39,000 rpm for 20 h at 4°C on a SW 41 Ti rotor (Beckman). After centrifugation the DRM fraction (interface of 5%/35% sucrose) was carefully collected, rinsed in 20mM PIPES, pH 7, 250mM sucrose, 1M EGTA and recentrifuged twice (100,000 g, 60 min, 4°C). The resultant pellet was resuspended with above buffer and stored at -80°C until use.

Evaluation of γ-secretase activity in cell culture

To assess the effect of elevated temperature on Aβ production, HEK293 cells stably expressing human APP_{695} KM670/671NL (Swedish) were plated at $1X10^6$ cells/9 cm^2 well; after 24h, cells were washed with serum-free media and medium refreshed (1ml). Cells were immediately placed

at 37 or 42°C and after 1h incubation the extracellular media was collected for Aβ ELISA analyses and cell viability was assessed (trypan blue staining). To determine the effects of mutations in APP on Aβ production, wild type HEK293 cells were transfected with pSG5-based expression vectors bearing wild type or mutant APP_{C99}3xFLAG cDNAs. At 36h post-transfection, cells were rinsed and medium refreshed. Extracellular media was collected after 4h incubation at 37°C. For all experiments, cells were cultured in Dulbecco's modified Eagle's medium/F-12 containing 10% fetal bovine serum and sAβ were analysed by MSD ELISA.

γ-Secretase *in vitro* thermo-activity assays

Proteolytic reactions were performed using purified ~10nM PSEN1/APH1A γ-secretase complexes and purified recombinant FLAG tagged substrates in 0.25% CHAPSO, 2.5% DMSO (or 10μM GSM, 100μM Fenofibrate), 0.1% Phosphatidylcholine, 150mM NaCl and 25mM PIPES over a temperature gradient ranging from 37°C-65°C for 20 min (except for the severe P88L mutant protease which activity was measured after 1h incubation and with 10X more enzyme). Enzyme mixes (containing all components except the substrate) and substrate dilutions were pre-incubated separately at the indicated temperatures for 10 min. After pre-incubation, substrate was added to the enzyme mix and proteolysis proceeded for 20 min or 1h (P88L). Final substrate concentrations in assays were 1.75 μM C99-3xFLAG or 2μM Notch-3xFLAG unless otherwise indicated.

γ-Secretase thermo-activity assays using $Aβ_{43}$, $Aβ_{45}$ or $Aβ_{46}$ synthetic peptides as substrates and purified wild type γ-secretase were carried out as indicated above with the following modifications: synthetic Aβ peptides were diluted in DMSO and proteolytic reactions were incubated for 1 hour ($Aβ_{45}$ or $Aβ_{46}$) or 4h ($Aβ_{43}$) at the indicated temperatures.

Thermo-activity analyses using DRMs as source of enzyme were performed similarly, but reactions contained 0.6 μg/μl or 1 μg/μl protein for DRMs prepared from Hi5 insect cells overexpressing γ-secretase components or human brain samples, respectively. Assays were carried out for 20 min for DRMs derived from insect cells or 4h for DRMs prepared from human brain samples in saline MBS buffer (150mM NaCl, 25mM MES), 0.1% DMSO, 1mM EGTA and protease inhibitors (Complete, ROCHE) with purified substrates at saturating concentrations. Temperature gradients ranging from 37°C-60°C and 60°-80°C were set for DRM analyses. All thermo-activity assays were performed on a PCR thermocycler (Biorad T100).

Quantification of γ-Secretase endopeptidase activity

γ-Secretase endopeptidase products (ICD-3XFLAG) generated in the proteolytic assays were quantified by SDS-PAGE western immunoblot and/or MALDI-MS (see **MALDI-MS analysis)**. In the first case, reactions were mixed with one volume of methanol-chloroform (1:2, v/v) to

remove hydrophobic molecules (lipids, substrate and membrane proteins) and aqueous fractions containing ICD products were analyzed by SDS-PAGE western immunoblot using anti-FLAG M2 antibody and quantified with Odyssey infrared imaging system.

Quantification of Aβ production by ELISA

$A\beta_{38}$, $A\beta_{40}$ (except for quantification of $A\beta_{40}$ from proteolytic reaction using $A\beta_{43}$ as substrate, which was quantified using AlphaLISA Detection Kit from PerkinElmer according to manufacturer's protocol) and $A\beta_{42}$ product levels were quantified on Multi-Spot 96 well plates pre-coated with anti-$A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ antibodies obtained from Janssen Pharmaceutica 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 x with 200 µl/well washing buffer (PBS + 0.05% Tween-20). 25 µl of SULFO-TAG JRF/AbN/25 detection antibody diluted in blocking buffer was mixed with 25 µl of standards (synthetic human $A\beta_{38}$, $A\beta_{40}$, $A\beta_{42}$ peptides) or reaction samples diluted in blocking buffer and loaded 50 µl per well. For the quantification of Aβ peptides generated in activity assays using synthetic $A\beta_{43}$, $A\beta_{45}$ and $A\beta_{46}$ as substrates, reactions with no enzyme were loaded to determine background levels. After overnight incubation at 4°C plates were rinsed with washing buffer and 150 µl/well of the 2x MSD Read Buffer T (tris-based buffer containing tripropylamine, purchased from Meso Scale Discovery) was added. Plates were read immediately on MSD Sector Imager 6000. Aβ43 product levels were quantified with β-Amyloid 1-43 kit from IBL according to manufacturer's instructions. $A\beta_{38}$, $A\beta_{40}$ and $A\beta_{42}$ product levels in proteolytic reactions using DRMs were analyzed by MALDI-MS (see below).

Detection of Aβ product profiles in Urea gels

Aβ-products were analyzed in urea-based bicine/tris SDS–PAGE (Wiltfang et al., 2002). Gel thickness was kept at 0.75mm and the composition of the separation gel was as follows: 8M Urea, 11% T/5% C polyacrylamide, 0.4M H_2SO_4, 0.25% SDS, pH=8.1. Electrophoresis was conducted at constant 100V for around 2h, after that, gels were transferred to a PVDF membrane and western immunoblot with 82E1 antibody, biotinylated anti-mouse IgG and streptavidin-HRP was performed. Signals were detected using ECL chemiluminescence with Fujifilm LAS-3000 Imager.

AlphaScreen substrate competition assays to assess γ-Secretase-Substrate relative affinities

Acceptor M2-anti-FLAG beads (0,1 µl/reaction) and purified APP_{C99}-3XFLAG (20 nM final concentration in assay) were pre-incubated with increasing concentrations of purified untagged APP_{C99} substrate or synthetic Aβ peptides in buffer with DDM (0,04% final concentration in assay) for 1h at 37°C (Mix 1, M1). Simultaneously, Mix 2 (M2) containing purified biotinylated γ-secretase (1 nM final concentration in assay), Inhibitor X (10 µM final concentration in assay),

0.1% Phosphatidylcholine, 0.005% Lauryl Maltose Neopentyl Glycol (LMNG) and donor beads (0,1 µl/reaction) was prepared and pre-incubated at 37ºC. Competition assays started with the mix of M1 and M2, binding signals were detected after 4 h incubation in dark at 37^{0}C. The assay was done in 384-well OptiPlates (PerkinElmer) in a final volume of 25 µl. Biotinylation of γ-secretase was done by incubation of purified γ-secretase with a 30 fold molar excess of EZ-link Sulfo-NHS-SS-Biotin reagent (ThermoFisher Scientific) during 2 h on ice. Remaining free biotin reagent was removed by dialysis using dialysis units (Spectra/Por Float-A-Lyzer G2) with a 100kDa cutoff. Apparent equilibrium dissociation constants (Kd) for APP C99-3XFLAG and synthetic Aβ43, and Aβ40 substrates using wild type human γ-secretase (PSEN1/APH1a) were derived by nonlinear curve fitting (Y=(Top-Bottom)/(1+10^(X-LogIC50)) + Bottom) using GraphPad Prism 7.01 software. According to Cheng and Prusoff equation for competitive binding (Cheng and Prusoff, 1973), apparent Kd equilibrium= IC50/(1+([L]/Kd)), where L is the APPC99 substrate; when Kd is significantly higher than the concentration of substrate, the value for [L]/Kd approaches zero and the apparent Kd approximates the IC50 in the competition binding assay.

Assessment of intrinsic thermostabilities of wild type and FAD-linked mutant γ-secretase complexes

Purified PSEN1/APH1A γ-secretase complexes containing wild type or pathogenic PSEN1 (P88L, Y115H, M139V, L166P, R278I, E280A, G384A or L435F) were diluted to similar protein concentrations in 150mM NaCl, 25mM PIPES and 0.5% CHAPSO and incubated for 15 min at 37, 45, 55 and 65°C. Next, in order to separate the heat-aggregated fraction from the remaining soluble enzyme, samples were centrifuged for 20 min at ~200000 g. The supernatant was collected and subjected to SDS-PAGE electrophoresis. Western immunoblot was performed with anti- PSEN1-NTF and PSEN1-CTF antibodies to assess the gradual heat-induced aggregation of the distinct protease complexes.

Determination of relative thermostabilities for wild type and FAD-linked mutant γ-secretase complexes using Nanobodies

Recombinant antibodies from Camelidae (VHHs, Nanobodies (Nbs)) are conformational-sensitive probes that display relatively high thermostabilities (Ewert et al., 2002; Olichon et al., 2007). These features make them suitable tools to investigate thermal-induced unfolding of their targets. We used two conformational anti-γ-secretase Nbs: Nb4 binds to a not yet fully characterized non-linear epitope in the multimeric complex (1% CHAPSO), but does not bind to TX-100 dissociated subunits. Nb28 binds to a conformational epitope in the NCT subunit. 10 nM purified, biotinylated wild type or mutant γ-secretase complexes were pre-incubated with 10 nM purified, His-tagged Nb4 or Nb28 in the presence of Streptavidin coated donor beads and Ni-chelate acceptor beads in assay buffer (25 mM Pipes, 150 mM NaCl, 0.5% CHAPSO and 0.1%

BSA) for 45 minutes at 30°C. The mix was split over 8 different PCR tubes (25µl/tube), which were incubated at the indicated temperatures for 1h, followed by 10 minutes incubation at at 25°C (thermal equilibration prior to binding detection). Temperature gradients ranging from 30°C-50°C and 50°-75°C were set on a Biorad T100 PCR thermocycler. Next, 20µl sample was transferred to a 384-well OptiPlate (PerkinElmer). Upon illumination, a luminescence signal at 620 nm was detected by the EnVision plate reader (PerkinElmer).

MALDI-MS sample preparation and analysis of γ-secretase products

Sample preparation for MALDI-TOF MS *in vitro* Aβ/AICD profiling was performed as follows: A total volume of 15 µl of *in vitro* γ-secretase activity reactions were mixed with 15 µl SA (38 mg/mL in water/ACN/TFA 40/60/0.2 (v/v/v)) without any additional purification or enrichment steps. Thereafter, 1 µl (8 technical replicates) of the matrix-analyte mix was applied on top of the thin SA layer using the dried droplet preparation (double layer) and air dried. All mass spectra were acquired on an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a 2 kHz Smartbeam™ laser using the AutoXecute function of the FlexControl 3.4 acquisition software: Briefly, each spectrum was acquired in linear positive mode within the mass range of *m/z* 2,500 to 20,000 with a low mass gate at *m/z* 2,000. 5,000 laser shots were automatically accumulated for each sample by random walk. Mass spectrometer parameters were balanced for optimal resolution and sensitivity in the Aβ peptide mass range (4-5 kDa). Subsequently, protein mass spectra were baseline-subtracted and externally calibrated in Flex Analysis 3.4 (Bruker Daltonics) using the protein calibration standard I (Bruker Daltonics). Average MALDI MS profiles were generated from eight single spectra using ClinProTools 3.0 software (Bruker Daltonics) (CPT). The following modified CPT processing parameters ((Munteanu B. and C. Hopf, 2016)) were used: Resolution 1000: Convex Hull baseline subtraction with a baseline flatness value of 0.75: Mass Range (*m/z*) 2,500-20,000: Spectra were recalibrated, allowing a mass tolerance of 500 ppm matched on 30 % of the peaks. Not recalibrated spectra were excluded. Peak picking was performed on total average spectra (based on intensity calculation and zero level integration type) using an intensity signal to noise (S/N) threshold > 5.

High Resolution MALDI FT-ICR

Monoisotopically resolved protein signals were recorded using a 7T Solarix XR MALDI FT-ICR mass spectrometer equipped with an Apollo II dual MALDI/ESI ion source and a 2 kHz Smartbeam II laser (Bruker Daltonics). Sample preparation for MALDI-FT-ICR measurements was done as previosly described. Data were acquired in positive ion mode from 150 to 10,000 *m/z* using magnitude mode and 4M data size with a resolving power of 390,000 at *m/z* 400 and ~40,000 at *m/z* 4,000, respectively, and a free induction decayof 2.9 s. The following tuning

parameters were used; Ion transfer (Funnel 1 100 V; Skimmer 1 45 V; Funnel RF Amplitude 150 Vpp; Octopole 2 MHz, RF Amplitude 350 Vpp; RF Frequency 1.4 MHz, 1200 Vpp; Transfer Optics Time of Flight 2.9 ms, Frequency 1 MHz, FR Amplitude 350 Vpp; Q1 Mass 4,000 *m/z*. Excitation Mode (Sweep Excitation; Sweep Step Time 15 µs), Ramped Power Excitation (Continuous, 14-28 %). A total accumulation of 100 scans was done. Before measurement the MALDI-FT-ICR MS was externaly calibrated using the Bruker protein calibration mix I containing the following components (Ubiquitin $[M+H]^+$ *m/z* 8560.623989, Insulin $[M+H]^+$ *m/z* 5730.608146 and the signal corresponding to the doubly charged Ubiquitin $[M+2H]^{2+}$ *m/z* 4280.815633). Spectra recalibration was done by performing a one-point intenal recalibration on the most promiment amyloid beta signal in the analysis and was done using the software Data Analysis 4.4 (Bruker Daltonics). Computational analysis and mass matching were performed using the Bruker Biotools 3.2 SR5 (Bruker Daltonics) software.

Elevation of body temperature in mice

Eleven to sixteen-week-old female APP NL mice were injected intraperitoneally with 30 µg of LPS (Sigma-Aldrich) in 500 µl of sterile saline. Age-matched control were treated simultaneously with saline only. Mice injected with LPS were immediately placed in cages preheated with a heating pad, a red light lamp and a 60 W bulb to ~ 38.5°C. Mice core body temperature raised within 25 min to 40°C (± 0.5°C) and then maintained at 39.9°C (± 0.6°C) for 100 min (**Figure S5**). Mice rectal temperature was monitored every 25 min (see **Figure S4)**. Control mice were kept at room temperature (RT, 22-24°C) and subjected to the same handling. At the end of the experiment mice were sacrificed in CO2 and decapitated. Collected blood was used to isolate plasma by 10 min centrifugation at 1500 g. $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ steady-state levels in plasma were analyzed by ELISA (multiplex MSD technology). The protocol was approved by ethical committee of KU Leuven (project number: 142/2015).

Statistical analysis

All statistical analysis was performed using GraphPad Prism software. Statistical evaluation of MALDI-MS data of drug/temperature effects on γ-secretase Aβ/AICD activity, were done on total ion count normalized (CPT) average mass spectra. Briefly, eight single mass spectra were processed as described above, and average intensity values (arbitrary units) were extracted for defined Aβ peptides or AICDs within a mass tolerance window of 500 ppm and transferred to GraphPad Prism software for further analysis and visualization. Linear MALDI TOF MS signals corresponding to amyloid beta were confirmed by high resolution MALDI-FT-ICR analysis with a mass accuracy **<<5ppm.**

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