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Molecular determinants and thermodynamics of the amyloid precursor protein transmembrane domain implicated in Alzheimer's disease

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Abstract

The deposition of toxic amyloid- β peptide (A β) aggregates in the brain is a hallmark of Alzheimer's disease. The intramembrane proteolysis by γ -secretase of the amyloid precursor protein carboxy-terminal fragment (APP- β CTF) constitutes the final step in the production of A β s. Mounting evidence suggests that APP- β CTF is a transmembrane domain (TMD) dimer, and that dimerization might modulate the production of A β species that are prone to aggregation, and therefore most toxic. We combined experimental and computational approaches to study the molecular determinants and thermodynamics of APP- β CTF dimerization, and produced a unifying structural model that reconciles much of the published data. Using a cell assay, which exploits a dimerization-dependent activator of transcription, we identified specific dimerization-disrupting mutations located mostly at the N-terminus of the TMD of APP- β CTF. The ability of selected mutants to disrupt the dimerization of full length APP- β CTF was confirmed by fluorescence resonance energy transfer experiments. Free-energy estimates of wild-type (WT) and mutants of the TMD of APP- β CTF derived from enhanced molecular dynamics simulations showed that the dimeric state is comprised of different arrangements, in which either ⁷⁰⁹GXXXXA⁷¹³ or ⁷⁰⁰GXXXG⁷⁰⁴GXXXG⁷⁰⁸ interaction motifs can engage in symmetric or asymmetric associations. Mutations along the TMD of APP- β CTF were found to modulate the relative free energy of the dimeric configurations, and to differently affect the distribution of interfaces within the dimeric state. This observation might have important biological implications, since dimers with a different arrangement of the transmembrane helices are likely to be recognized differently by γ -secretase and lead to a variation of A β levels.

Keywords

molecular dynamics; TOXCAT; FRET; metadynamics; amyloid- β peptide

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Introduction

The neuropathological hallmarks of Alzheimer's disease (AD) include the presence of senile plaques and neurofibrillary tangles throughout the cerebral cortex and hippocampus¹. These plaques, which are composed mainly of dystrophic neurites surrounding extracellular aggregates of amyloid β -peptides (A β s), have been hypothesized to initiate a pathological cascade that can result in cognitive decline^{2,3}. The sequential proteolytic degradation of amyloid precursor proteins (APPs) produces A β peptides that range from 34 to 42 amino acids in length^{3,4}. The ectodomain of APP is first proteolyzed by the β -site of APP cleaving enzyme 1 (BACE1). The resulting C-terminal fragment (APP- β CTF; amino acid residues 672-770 according to the residue numbering of the APP770 isoform) is subsequently cleaved within its transmembrane domain (TMD) by γ -secretase, a multisubunit integral membrane enzyme composed of presenilin, nicastrin, anterior pharynx defective 1 (APH-1) and presenilin enhancer 2 (PEN-2)⁵.

γ -Secretase can cleave the TMD of APP- β CTF at multiple sites (commonly referred to as the γ -, ϵ - and ζ -sites). The ϵ cleavage site closest to the cytoplasmic border of the TMD releases the intracellular domain of APP- β CTF into the cytosol⁶. The remaining transmembrane fragment undergoes an intermediate scission, ~3 residues N-terminal to the ϵ -site, at the ζ -site⁷. Thereafter, A β is released by final cuts at the γ -site. The γ -cleavage is variable and it can occur after A β amino acid residues 38, 40 or 42. The balance between these γ -cleavages can have an important role on the pathogenicity of A β s. As the longer peptide A β 42 - relative to the A β 40 species - is prone to form aggregates, an increase in the A β 42/40 ratio appears to be associated with the pathogenesis of early-onset familial AD⁸. Indeed, in normal individuals APP processing leads to low A β 42/40 ratios. In contrast, individuals with mutations in presenilin or in APP can have elevated A β 42/40 ratios⁹⁻¹⁷, which account for ~50% of cases of familial AD. Currently, the mechanism underlying the promiscuity in the cleavage of the TMD of APP- β CTF by γ -secretase and the regulation of the different levels of A β s is not understood.

Recently, the possibility has been raised that the oligomeric state of the substrate APP- β CTF modulates A β production, and more specifically the A β 42/40 ratio. The TMD region of APP- β CTF (residues 700-723), which contains two consecutive GXXXG motifs (⁷⁰⁰GAIIG⁷⁰⁴ and ⁷⁰⁴GLMVG⁷⁰⁸) known to mediate TMD interactions¹⁸⁻²⁵ through a combination of van der Waals packing²⁶ and C α -H...O hydrogen bonds²⁷, has been suggested to be oligomeric²⁸. Moreover, using a neuronal cell system Munter *et al.* showed that pairwise mutations of glycine residues within the first transmembrane ⁷⁰⁰GXXXG⁷⁰⁴ motif to alanine could gradually attenuate the dimerization propensity of the TMD of APP- β CTF²⁹. At the same time, these mutations appeared to reduce the production of A β 42, left the levels of A β 40 unaffected, and increased the levels of A β 38 and shorter A β species. In contrast, Kienlen-Campard *et al.*³⁰ found that pairwise replacement of the same two glycines by leucines or isoleucines, but not by alanines, led to a drastic reduction of A β 40 and A β 42 levels. Remarkably, instead of disrupting the dimerization of APP- β CTF, these mutations enhanced its dimer stability as measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)³⁰. In a more recent study, Gorman *et al.*³¹ concluded that the dimerization of the TMD of APP- β CTF was driven by a ⁷⁰⁹GXXXA⁷¹³ motif not involving either of the two glycines in the ⁷⁰⁰GXXXG⁷⁰⁴ region. In contrast, the recent solid-state NMR structure of the TMD of APP- β CTF³² suggested that dimerization was mediated by two consecutive ⁷⁰⁰GXXXG⁷⁰⁴ and ⁷⁰⁴GXXXG⁷⁰⁸ motifs. Thus, the precise transmembrane interactions involved in the oligomerization of APP- β CTF remain controversial. In addition, the dimeric nature of APP- β CTF has not been established directly, but rather inferred indirectly from experiments involving its TMD region.

Here we employed fluorescence resonance energy transfer (FRET) to show that purified APP- β CTF dimerizes in dodecyl maltoside (DDM) with a high-affinity and through specific transmembrane helix-helix interactions. Phenylalanine scanning-mutagenesis performed with the TOXCAT oligomerization assay³³ indicated that the dimerization interface is complex and involves the consecutive ⁷⁰⁰GAIIG⁷⁰⁴ and ⁷⁰⁴GLMVG⁷⁰⁸ sequences of the TMD of APP- β CTF, as well as amino acid residue V715 located between the γ - and ϵ -cleavage sites. Free-energy estimates were obtained from coarse-grained (CG) enhanced molecular dynamics (MD) simulations of both WT (residues 699-724) and mutant APP- β CTF TMD helices in an explicit dipalmitoyl phosphatidylcholine (DPPC)-water environment, using the well-tempered metadynamics algorithm. These computational results recapitulate the experimental TOXCAT data, and show that the simulated APP- β CTF TMD helices can attain different dimeric configurations characterized by markedly different crossing angles, and residue composition.

Results

The TMD mediates APP- β CTF dimerization

Although recent reports have suggested that the TMD of APP- β CTF is dimeric, the oligomeric state of full length APP- β CTF has not yet been established. To this end, we applied a strategy that combines SDS-PAGE analysis and FRET. We employed a recombinant form of APP- β CTF (Figure 1A) designed originally by Li *et al.*³⁴, which encodes the entire APP- β CTF region followed by a C-terminal Flag-tag. This construct constitutes a popular substrate to assay γ -secretase activity, both *in vitro* and *in vivo*, and it has been shown to yield physiological A β 42/A β 40 ratios³⁴⁻³⁶. APP- β CTF was expressed in *E. coli*, extracted from cytoplasmic membranes using the detergent DDM and purified using anti-Flag affinity chromatography. Oligomerization was assayed by SDS-PAGE, a method employed extensively to probe transmembrane helix-helix interactions in single-span proteins such as Glycophorin A (GpA)¹⁸, phospholamban,³⁷ and BCL2/adenovirus interacting protein 3³⁸. As shown in Figure 1B, a significant fraction of APP- β CTF migrated as a dimer with an apparent molecular mass of 27 kDa. To further establish the dimeric nature of APP- β CTF we employed electron microscopy. A APP- β CTF cysteine variant (S730C) was purified in DDM. This new cysteine is 7 amino acid residues away from the cytoplasmic border of the TMD, in a location that does affect the dimerization observed by SDS-PAGE (not shown). In addition, this cysteine appears to be highly water accessible and outside the region interacting with the detergent micelle, as indicated by a value of 0.95 molar equivalent of accessible sulfhydryl groups determined with DTNB³⁹. The APP- β CTF variant was labeled with a conjugated maleimide-nanogold chemical probe (Nanobrobes, Yaphank, NY) that specifically reacts with cysteines. The gold-labeled APP- β CTF was imaged by negative stain electron microscopy and found to be mostly dimeric on the grid (Figure S1). We employed this same S730C APP- β CTF variant for our FRET experiments in the detergent DDM. For FRET we labeled this single cysteine with the fluorescence pair IAEDANS (energy donor) and IANBD (energy acceptor). The chemical composition of these fluorescent probes is reported in the Materials and Methods section. We have used this same FRET pair elsewhere to monitor membrane protein dimerization⁴⁰, and it offers a good spectral overlap⁴¹ and an optimal R_0 of 27 Å⁴². The iodoacetamide reactive groups of these fluorescence probes allowed for their incorporation into the single cysteine APP- β CTF variant with stoichiometries of 0.9 and 0.8 for IAEDANS and IANBD, respectively. The reaction with the sulfhydryl group was highly selective as judged from the low levels of incorporation of the fluorescent probes into the native protein (<7% modification under identical labeling conditions). For AEDANS-APP- β CTF peak fluorescence excitation and emission intensities were observed at 339 and 445 nm, respectively. For ANBD-APP- β CTF the values were 492 nm for the peak excitation

intensity and 530 nm for the peak emission intensity. FRET between the donor and acceptor was measured by the donor quenching method using an excitation wavelength of 339 nm and recording spectra in the 400-600 nm range (Figure 1C). A large quenching of the donor emission was observed in the presence of acceptor as evidenced by the decrease in fluorescence intensity at 462 nm, which resulted in an E of ~59%, indicating oligomerization of the labeled protein. The plot shown in Figure 1D (black circles) indicates that the affinity for dimerization is high with a $K_D=0.2 \mu\text{M}$. To avoid potential artifacts due to protein crowding within a given micelle the fraction of micelles over protein molecules in our FRET experiments is >10 fold.

To ascertain that APP- β CTF dimerization is driven by its TMD and not by flanking regions, we constructed a chimeric protein (termed MBP-APP- β CTF) consisting of maltose binding protein MBP followed by the TMD of APP- β CTF (residues 700-722), and a C-terminal hexa-histidine tag (Figure 1A). A cysteine variant of this chimera (S421C) was expressed from a pET vector in *E. coli*, purified in DDM using nickel-nitrilotriacetic acid (Ni-NTA) chromatography and labeled at this cysteine with either IAEDANS or IANBD. The oligomerization of this chimera was measured by FRET in an analogous manner as described above. A large quenching of the donor emission was observed (Figure S2B) in the presence of acceptor as evidenced by the decrease in fluorescence intensity at 462 nm (an E of ~55%), indicating dimerization of the labeled protein. The dimerization affinity of MBP-APP- β CTF was comparable to that displayed by APP- β CTF (Figure 1D, gray circles). Finally, to demonstrate that the APP- β CTF dimerization was indeed TMD specific, we carried out a competition experiment in which MBP-APP- β CTF was titrated to a mixture of fluorescence labeled APP- β CTF (Figure 1E). As we increased the concentration of MBP-APP- β CTF, the 1 : 4 AEDANSAPP- β CTF : ANBD-APP- β CTF dimer dissociated, as reflected by a decrease in donor quenching. The observed competition by MBP-APP- β CTF indicated that APP- β CTF dimerization was indeed mediated by its TMD.

Transmembrane sequence specificity of APP- β CTF dimerization

To investigate the transmembrane sequence specificity of APP- β CTF dimerization we employed the TOXCAT assay, which reports on TMD homo-oligomerization in the cytoplasmic membrane of *E. coli*³³. This assay has been used extensively to probe transmembrane helix interactions in the context of a biological membrane^{20; 25; 38; 43-45}. The TOXCAT assay exploits a dimerization-dependent activator of transcription, termed ToxR (Figure S2A). The TMD sequence of interest is expressed in *E. coli* as a chimeric protein flanked by ToxR and by MBP. TMD-mediated oligomerization results in ToxR-activated expression of a reporter gene encoding chloramphenicol acetyltransferase (CAT). The level of CAT activity correlates with the strength of a given TMD association³³. Figure 2A depicts the sequences that were inserted in the TOXCAT chimera. WT GpA is known to form a strong homodimer³³, and thus was used to set a baseline for comparison (Figure 2B). In contrast the G83I mutant of GpA, long established to weaken GpA dimerization, was used as a calibration of weak interactions. In this system, noninteracting TMDs give signals close to background CAT activity, less than 1% of WT GpA, while the G83I variant displays ~10% GpA activity⁴⁶. The TMD of APP- β CTF displayed approximately 40% CAT activity when compared to GpA (Figure 2B), indicating a relatively strong association in the cytoplasmic membrane of *E. coli* that is comparable to the signal reported for the TMD association of some single-pass receptors²⁵. We routinely employed established controls for the TOXCAT assay³³ to measure the expression level of the chimeras, and their correct topology. Specifically, anti-MBP western blot analysis showed comparable expression levels for the mutant chimeras and maltose complementation assays indicated the chimeras inserted with the right topology (not shown).

An effective strategy to probe the specificity of TMD interactions is to introduce steric clashes or to alter the ideal packing by changing the size of amino acid residue sidechains¹⁸. To investigate the sequence specificity of the TMD dimerization of APP- β CTF we substituted, one at the time, each amino acid residue in the TMD to phenylalanine and measured the TOXCAT signal of each protein variant. We chose phenylalanine because it has been successfully used in the TOXCAT assay to disrupt the dimerization interface of GpA³³, and since it is often found in the hydrophobic region of transmembrane helices^{47; 48}, we did not anticipate expression or folding problems. As shown in Figure 2C, phenylalanine substitution along the APP- β CTF TMD strongly modulated the TOXCAT signal, relative to the signal displayed by the WT sequence. To demonstrate that the scanning mutagenesis results were not exaggerated by the choice of a bulky phenylalanine as a substituting amino acid residue, we also considered familial AD mutants V717G/L/I⁴⁹⁻⁵⁵. Notably, the phenylalanine substitution at position V717 was less disruptive of association than substitution by the smaller Gly (see Figure S3).

The amino acid sequence dependence of the TOXCAT signal can be separated into three groups: those amino acid positions where substitution to phenylalanine had a strong effect on CAT activity (CAT signal between 0-0.1 relative to the WT TMD sequence of APP- β CTF; positions: 700, 701, 702, 703, and 715); those positions in which mutation to phenylalanine had a partial effect (CAT signal between 0.1-0.6 relative to the WT TMD sequence; positions: 704, 705, 706, 707, 708, 710, 712, 714, 716, 718, 720, 721, and 722); and those positions in which the effect was marginal (CAT signal between 0.6-1.0 relative to the WT TMD sequence; positions: 709, 711, 713, 717, and 719). We also addressed the question of whether the sequence specificity for the dimerization of the TMD of APP- β CTF derived from the TOXCAT assay was also relevant for the full-length APP- β CTF. To this end, we selected the mutations G700F and V715F that were predicted by the TOXCAT assay to disrupt the dimerization of APP- β CTF. The variants G700F and V715F of APP- β CTF were produced and assayed for their ability to dimerize using the FRET assay described above. As displayed in Figure S2D-E, each of these TMD mutations disrupted the dimerization of APP- β CTF significantly, indicating a correlation between our TOXCAT data and the dimerization of full-length APP- β CTF. However, we note that relative to the WT sequence, a significant energy transfer was still apparent for these two variants. Indeed, partial association in detergent micelles, even for mutants that show a strong disruption in the TOXCAT assay is not uncommon. Glycophorin A (GpA) mutants that are strongly disruptive as evaluated by TOXCAT can give a significant association in the detergent SDS³³. Conversely, mutations that are partially disruptive in the TOXCAT assay can almost abolish dimerization in SDS³³. Thus, the data derived from these two assays should be compared from a qualitative perspective.

Structural models of APP- β CTF TMD dimer

To rationalize the TOXCAT results from a structural and energetic perspective, we studied the dynamic behavior of interacting APP- β CTF TMD helices, in either WT or mutant forms, using computational methods. Specifically, we carried out well-tempered metadynamics simulations of CG representations of the WT TMD of APP- β CTF (segment K699-K724) in an explicit DPPC-water environment (see Materials and Methods section for details). We then compared its resulting free-energy profile to those obtained for several mutant forms of the TMD of APP- β CTF (see following section for results of simulations carried out on mutants G700F, A701F, I702F, I703F, G709F, A713F, V715F, and V717F/G/L/I). Simulations were performed using as reaction coordinates the distance (d) between the centers of mass (COMs) of interacting TM helices, and the crossing angle (Ω) defined by the COMs of the cyto- and ecto- halves of the two interacting helices (Figure 3). A negative value for this crossing angle implies a so-called “right-crossing” conformation, whereas a

positive value indicates a “left-crossing” conformation. To facilitate fast convergence of these simulations, we used the multiple walker approach starting from four different model structures of the TMD of APP- β CTF (see Materials and Methods section for details).

Figure 4 displays the free-energy profile we obtained for the WT system as a function of the chosen reaction coordinates (d and Ω). To speed up convergence in the relevant region of the phase space, i.e. the region containing APP- β CTF TMD dimers, we restricted the simulations to conformations with $d \leq 1.9$ nm by adding a strong repulsive potential above this threshold. Notably, the explored region also contained non-interacting helices, as evinced by the lack of angular dependence of the mean force at distances above 1.5 nm. Visual inspection of the conformations sampled in this region of the phase space confirmed that the two helices were separated by at least four layers of lipids. The dimeric conformations appear to span a large region of the crossing angle space, extending from right-crossing extreme configurations at $\Omega \sim -30^\circ$ to parallel orientations of the helices with respect to one another at $\Omega \sim 0^\circ$. The absolute minimum is located at $d = 0.72$ nm and $\Omega = -28^\circ$ and is labeled as *A* in Figure 4. A secondary, smaller basin (*B* in Figure 4) is identified at $d = 0.82$ nm and $\Omega = -10^\circ$, and is ~ 2 kcal/mol less stable. Left-crossing extreme configurations ($\Omega \sim 20^\circ$) are also explored, but appear energetically less stable.

To investigate the structural composition of the most stable basins *A* and *B*, we clustered their constituent configurations according to the level of rotation of the helices along their axis (see azimuthal angles α_1 and α_2 in Figure 3), and the consequent orientation of the $^{700}\text{GXXXG}^{704}\text{XXXG}^{708}$ interfaces on each helix (see Materials and Methods section). Figure 5 shows the representative dimeric configurations of these clusters. Analyzing the combined distribution of the combined values of the crossing and azimuthal angles we estimate that the most abundant class of configurations (72%) of the APP- β CTF TMD dimer consists of physically indistinguishable “anti-symmetric” interfaces. Azimuthal angles fluctuating between 40° and 70° (A1 in Figure 5), and between 130° and 160° in (A2 in Figure 5) characterize these interfaces. Contact maps calculated as an average over the conformations of cluster A1 or cluster A2 provided further details of these anti-symmetric interfaces. As shown in Figure S4, the dimer interface of the most abundant class of configurations (e.g., those of basin A1) is mostly formed by the $^{709}\text{GXXXA}^{713}$ motif rather than the $^{700}\text{GXXXG}^{704}$ motif. The inter-helical contacts (defined as pairs of residues for which the minimum distance between the CG beads is below 5.5 Å) I702-I'703, L705-V'707, L705-M'706, M706-M'706, G708-V'707, G709-V'707, G709-V'710, G709-V'711, V710-V'710, A713-T'714, A713-V'711, A713-V'715, V717-V'715, and V721-M'722 are constituent parts of this interface. Two “symmetric” classes of dimeric configurations are also present in basin A, and these are also characterized by an interface involving the $^{709}\text{GXXXA}^{713}$ motif with the $^{700}\text{GXXXG}^{704}\text{XXXG}^{708}$ motif lying either on a side proximal to the $^{709}\text{GXXXA}^{713}$ interface (cluster A3 in Figure 5) or distal to it (cluster A4 in Figure 5). In the former case (cluster A3), the dimeric configuration is characterized by contacts L705-L'705, A713-A'713, and V717-V'717. On the other hand, contacts involving residues V710 or T714 determine the dimeric association in configurations of cluster A4. The clusters in the secondary basin B are characterized by more parallel orientations of the interacting helices, with contacts in the region of the $^{700}\text{GXXX}^{704}\text{GXXXG}^{708}$ motif (see clusters B1 and B2 in Figure 5). Specifically, cluster B1 (see also Figure S5, left panel) is characterized by the symmetric hydrophobic contacts G700-G'700, G704-G'704, G708-G'708, I712-I'712, and I715-I'715, in addition to contacts G700-A'701, A701-K'699, A701-I'703, A701-G'704, I703-A'701, G704-A'701, G704-V'707, L705-V'707, V707-G'704, V707-L'705, G708-V'711, I712-V'715, and V715-I'712. On the other hand, the conformations in cluster B2 (see Figure S5, right panel) correspond to asymmetric APP- β CTF TMD dimers stabilized by contacts V705-I'703, G709-V'710, I716-V'714, and L720-I'718.

Correlation between TOXCAT data and structural models

To better compare the computational results with the experimental data, we simulated mutants G700F, A701F, I702F, I703F and V715F, in view of their ability to strongly affect the stability of the APP- β CTF TMD dimer (more than 90% reduction in CAT activity relative to the WT TMD of APP- β CTF). As a control, we also simulated the G709F, A713F, and V717F/G/I/L familial AD mutants, which did not show significant reduction in the TOXCAT signal and thus were inferred not to affect the stability of the APP- β CTF TMD dimer. We integrated the free-energy dependence on the crossing angle, and obtained the one-dimensional free energy profiles for all aforementioned variants (Figure 6). Taking the conformation at $d=1.9$ nm as a reference state for non-interacting helices, we calculated the variation of dimeric stability upon mutation ($\Delta\Delta G = \Delta G(\text{Mutant}) - \Delta G(\text{WT})$), and reported these values in Table 1. All simulated mutant systems exhibited a positive $\Delta\Delta G$ value, indicating a less favorable dimeric state. However, mutants A713F, G709F, and V717F/G/I/L which had resulted in a CAT production similar to WT, exhibited only a slight increase ($\Delta\Delta G \sim 0.3\text{--}0.7$ kcal/mol) in the free-energy, in contrast to the more pronounced ($\Delta\Delta G > 2.5$ kcal/mol) destabilizing effect of the APP- β CTF TMD dimer noted for mutants that had resulted in significant reduction of CAT expression.

Comparison between the two-dimensional free-energy profiles of WT and the mutants (see Figures S6 and S7) provides insight into the structural reasons underlying the loss of stability of these variants of APP- β CTF TMD. Based on the profiles of Figure S6, it appears that destabilization of the symmetric configurations of cluster *B* (especially B1) with the $^{700}\text{GXXXG}^{704}\text{XXXG}^{708}$ motif at the interface is the main difference with respect to the free-energy of the WT TMD of APP- β CTF, and thus a possible reason behind the inferred dimerization disruption. In the G700F mutant, the bulky side-chain in position 700 breaks the symmetric G700-G'700 and G700-A'701 interactions, also displacing the A701 residue, and preventing the formation of the A701-I'703 and A701-G'704 contacts. As a consequence, the free-energy in the region where basin *B* is located in the WT APP- β CTF TMD system does not display a minimum for the G700F mutant (compare panels A and F in Figure S6 corresponding to the free-energy surfaces of G700F and WT, respectively), while a basin (labeled A in Figure S6) containing right-crossing conformations is maintained, with roughly the same structural composition of its WT counterpart (75% asymmetric configurations for clusters A1 and A2, and 25% symmetric configurations for clusters A3 and A4). The same effect can be observed from the free-energy maps of mutants A701F, I703F and V715F (Figure S6 panels B, C, and D, respectively). The loss of interactions between A701 and I'703 or G'704, characteristic of the configurations of the B2 cluster, upon A701F mutation might cause the absence of the B basin in the free-energy map deriving from the A701F mutant simulations (Figure S6, panel B). A similar effect is responsible for the results obtained for the I703F mutant (Figure S6, panel C). Mutation at this position also causes disruption of the I702-I'703 contact, which contributed to the stabilization of the dimeric configurations of the A1/A2 clusters in the WT, thus affecting the free energy of basin A. In a similar fashion, the V715F mutant (see Figure S6, panel D) has a reduced contribution of the B basin. The case of the V702F mutant (Figure S6, panel E) constitutes the only exception to this trend. The loss of the I702-I'703 contact destabilizes the asymmetric $^{709}\text{GXXXXA}^{713}$ state. Similar to the case of the V703F mutant (Figure S6, panel C), the prevalence of the asymmetric $^{709}\text{GXXXXA}^{713}$ dimers is significantly reduced, while the dimers in cluster B2 retain relevance in the dimerization population, as supported by the depth of the respective free-energy basins (A and B in Figure S6, panel E). Notably, although the simulated mutants that exhibited CAT expression similar to WT, i.e., G709F, A713F, and V717F/G/I/L exhibit these positions at the interface in the conformations in the right-crossing basin A (see Figure 4 and clusters A1 and A2 in Figure 5), the $^{709}\text{GXXXXA}^{713}$ interface is not affected by substitutions at these positions, as evident in the free-energy

surfaces reported in Figure S7. These surfaces show clear minima in the location of basins A and B, although the relative depth of basin B with respect to basin A is increased for these mutants.

Discussion

Understanding the oligomeric structure of the γ -secretase substrate APP- β CTF is likely to provide relevant insight into the molecular mechanisms underlying the cleavage site promiscuity displayed by γ -secretase, and the resulting variation in A β s levels. Previous attempts to study the transmembrane helix-helix interactions involved in APP- β CTF oligomerization have yielded somewhat conflicting results. A study employing the ToxR assay⁵⁶, which is analogous to the TOXCAT assay³³ employed here, suggested that the glycine residues within the first transmembrane ⁷⁰⁰GXXXG⁷⁰⁴ motif modulated the dimerization propensity of the TMD of APP- β CTF²⁹. However, a rather modest extent of oligomerization disruption was achieved when each of these glycines was mutated²⁹. In our studies, mutation of the aforementioned G700 and G704 to phenylalanine had a strong destabilizing effect on dimerization. Our TOXCAT data also suggested that the dimerization interface was complex and that the second consecutive ⁷⁰⁴GXXXG⁷⁰⁸ was pivotal to the stabilization of the TMD dimer. This observation is in good agreement with the recent solid-state NMR structure of the TMD of APP- β CTF³², which suggested that dimerization was mediated by two consecutive ⁷⁰⁰GXXXG⁷⁰⁴ and ⁷⁰⁴GXXXG⁷⁰⁸ motifs. Alternatively, Gorman *et al.*³¹ employed FRET and SDS-PAGE to conclude that the dimerization of synthetic peptides of the TMD of APP- β CTF was solely driven by a ⁷⁰⁹GXXXA⁷¹³ motif. Our phenylalanine scanning mutagenesis did not reveal the involvement of this motif in dimerization. However, our MD simulations clearly emphasized the important role of the ⁷⁰⁹GXXXA⁷¹³ motif in the dimeric interaction.

Unlike previous computational studies of the APP- β CTF TMD dimer^{29; 30}, which employed computational searches for low energy conformations in vacuum⁵⁷, our simulations were carried out in an explicit - albeit CG - membrane environment, and allowed to obtain relative free-energy estimates for the WT and mutant systems. Our free-energy calculations of the WT APP- β CTF TMD suggested the formation of 72% asymmetric dimer configurations with ⁷⁰⁹GXXXA⁷¹³ at the interface, 16% symmetric configurations with ⁷⁰⁰GXXXG⁷⁰⁴GXXXG⁷⁰⁸ at the interface, 8% symmetric configurations with ⁷⁰⁹GXXXA⁷¹³ at the interface, and 4% asymmetric configurations with ⁷⁰⁰GXXXG⁷⁰⁴GXXXG⁷⁰⁸ at the interface. Support for the formation of different APP- β CTF TMD dimeric interfaces was already reported in the literature using either a MD simulated annealing simulation in vacuum³², or implicit membrane replica exchange simulations⁵⁸. We note that our symmetric configurations with ⁷⁰⁰GXXXG⁷⁰⁴GXXXG⁷⁰⁸ at the interface resemble the symmetric ⁷⁰⁰GXXXG⁷⁰⁴GXXXG⁷⁰⁸-mediated dimeric structures obtained by simulated annealing³². We note however, that the presence of an explicit membrane environment in our simulations imposes a specific orientation to the charged residues at the APP- β CTF TMD helix boundaries, which determines a more parallel configuration of the dimer.

Our studies also provide specific estimates of the absolute dimerization free energy of WT APP- β CTF TMD. While the apparent dimerization constant obtained from our FRET experiments depends on the amount of detergent present in the sample, measuring concentrations as molar fractions in the hydrophobic phase of the sample, and defining a mole fraction dimerization constant can eliminate this dependence. Using our measured K_D and detergent concentration, we obtain a $\Delta G^\circ = -5.4$ kcal/mol. Notably, this value is in general agreement with the reported value measured in sodium dodecyl sulfate (SDS) micelles³¹, which corresponds to $\Delta G^\circ = -4.5$ kcal/mol. Based on the results from the

mono-dimensional free-energy profile deriving from our simulations, we obtain a mole fraction standard free-energy in the bilayer of $\Delta G^\circ = -9.1$ kcal/mol. As expected, this value is larger in absolute terms when compared to the dimerization free energy in micelles. The difference between the geometry of the hydrophobic regions in a micellar environment and in the bilayer contributes to different entropies of orientation and translation, and accounts for the increased stability of the dimer in the lamellar geometry⁵⁹. Although the difference in free-energy is difficult to quantify, it is known to be of the order of a few kcal/mol for GpA dimers⁵⁹.

Finally, our results suggest a qualitative agreement between the dimerization free-energy estimates calculated for several mutants and the TOXCAT experimental data: mutations with a large positive $\Delta\Delta G$ also have a pronounced reduction in CAT expression, and vice versa, mutations with little effect on the CAT expression also show little changes in $\Delta\Delta G$. Although the qualitative nature of the TOXCAT assay does not allow us to derive estimates of free-energy differences from the experiments, and to compare them directly to our computational results, we note that the $\Delta\Delta G$ values obtained from our simulations are of the right order of magnitude³¹.

In summary, our results are consistent with the APP- β CTF TMD dimeric state being comprised of a number of different arrangements in which either the ⁷⁰⁹GXXXXA⁷¹³ or ⁷⁰⁰GXXXG⁷⁰⁴GXXXG⁷⁰⁸ motifs can be at the interface in symmetric or asymmetric associations. Mutations along the APP- β CTF TMD helices modulate the relative free energy of the dimeric configurations, and thus the relative free energy of the monomeric and dimeric states. Most importantly, mutations can affect differently the distribution of interfaces within the dimeric state characterized by markedly different crossing angles, and residue composition. Notably, comparison of representative structures reveals that the A β 42 site is more exposed, relative to the A β 40 site, in asymmetric configurations of the less stable basin of WT APP- β CTF. Conversely, the asymmetric configurations of the WT APP- β CTF lowest energy basin exhibit a more exposed A β 40 site relative to the A β 42 site. The relevance of this observation is yet unclear, but it does raise the tantalizing possibility that different arrangements of the transmembrane helices of APP- β CTF may be recognized and processed differently by γ -secretase, forming different complexes with it, and being cleaved at different positions. Overall, the combined experimental and computational approach employed here yields a unifying structural model that reconciles much of the published data, and provides relevant insight into the molecular determinants involved in the TMD oligomerization of APP- β CTF. This information might inspire rational design of small molecule ligands that might cause dimerization disruption.

Materials and Methods

Chemicals

Restriction enzymes and DNA modifying enzymes were from New England Biolabs. Oligonucleotides were purchased from (IDT technologies, Coralville). Triton X-100 and DDM were purchased from Anatrace. Fluorescent probes N,N-dimethyl-N-(iodoacetyl)-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD), 5-((2-iodoacetyl)amino)ethylamino)naphthalene-1-sulfonic acid (IAEDANS), Oregon green 488 maleimide and Texas red C2 maleimide were purchased from Molecular Probes (Invitrogen). All other chemicals were of the highest purity commercially available.

Expression and Purification of APP- β CTF

E. coli BL21(DE3) cells transformed with plasmid pET-21b (kindly provided by Dr. Li, Sloan-Kettering), encoding amino acid residues 596-695 of the 695-aa isoform of APP

(APP695) followed by the Flag sequence (DYKDDDDK)³⁴, were grown in enriched medium (0.85% Na₂HPO₄, 0.03% KH₂PO₄, 0.5g/l NaCl, 0.01% NH₄Cl, 10g/l Tryptone, 5g/l Yeast Extract, 2mM MgSO₄, 1mM CaCl₂, 0.6% Glucose, 10mg/ml Thiamine, 100 µg/ml Ampicillin) at 37°C and induced at A₆₀₀~0.6 with 0.4 mM isopropyl-β-D-thiogalactoside. Three hours after induction cells were harvested by centrifugation at 6,000xg for 10 minutes at 4°C. The pelleted cells were resuspended in lysis buffer (50mM Tris-HCl, 300mM NaCl, 20mM magnesium acetate, 1mM phenylmethylsulfonyl fluoride (PMSF), pH 8.0), incubated with 0.1mg/ml hen egg white lysozyme and 0.01 µg/ml DNase for 30 minutes on ice, and lysed by two passages over a French press at 1,000 psig. Unbroken cells and debris was removed by centrifugation at 10,000xg for 20 min at 4°C, and cytoplasmic membranes were isolated from the supernatant by centrifugation at 130,000xg for 1 h at 4°C. The membranes were resuspended in 100mM NaHCO₂, 1mM PMSF at pH 11.3, incubated for 30 min in ice and centrifuged again at 130,000xg for 1 h at 4°C. This procedure was repeated twice to remove peripheral membrane proteins. The washed cytoplasmic membranes were extracted in lysis buffer containing 2% Triton X-100 for 2 h at 4°C and the resulting solubilize centrifuged at 200,000xg for 1 h at 4°C to remove insoluble material. This supernatant contains detergent solubilized APP-βCTF. APP-βCTF was purified in a single chromatographic step over antibody affinity M2 resin (Sigma) and eluted according to the manufacturer's instructions in 0.1% DDM. Mass spectrometry confirmed the identity of purified APP-βCTF. Site directed mutagenesis was performed using the QuikChange kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). All sequences were confirmed by automated dideoxynucleotide sequencing.

Expression and Purification of Maltose Binding Protein Chimera

E. coli BL21(DE3) cells transformed with a pET plasmid containing MBP-APP-βCTF were grown, induced and harvested as above. Cells were resuspended in 1:50 culture volume of lysis buffer (50 mM Tris-HCl, 300mM NaCl, 1mM phenylmethylsulfonyl fluoride, pH 8.0), incubated with 0.1mg/ml hen egg white lysozyme and 0.1 µg/ml DNase for 30 minutes on ice, and disrupted by French press at 1,000 psig. The insoluble material was removed by centrifugation at 10,000xg for 20 min, and total bacterial membranes were isolated from the supernatant by ultracentrifugation at 200,000xg for 1 h. The pellet was extracted with lysis buffer plus 2% Triton X100 and subjected to ultracentrifugation. The MBP-APP-βCTF chimera was bound to Ni-NTA agarose resin (Quiagen, Valencia, CA) and eluted according to the manufacturer's instructions in 0.1% DDM. Mass spectrometry confirmed the identity of purified APP-βCTF-TMD.

Construction TOXCAT chimeras

A synthetic duplex DNA encoding the amino acid sequence of human APP695 TMD (Gly⁷⁰⁰-Met⁷²²) containing *NheI* and *DpnII* ends was cloned into the *NheI* and *BamHI* sites of the TOXCAT vector pccKAN (kindly provided by Dr. Engelman, Yale University). The resulting vector is referred to as pccAMP. The vector pccKAN, and its derivatives encoding for the TMD of Glycophorin A (GpA) (pccGpA-WT) and an oligomerization disruptive single amino acid mutant (pccGpA-G83I) have been described previously³³. Site directed mutagenesis was performed in pccAPP using the QuikChange kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). All sequences were confirmed by automated dideoxynucleotide sequencing.

Expression of TOXCAT chimeras and spectrophotometric CAT assay

Plasmids encoding the TOXCAT chimeras were transformed into *Escherichia coli* NT326 cells (kindly provided by D. Engelman) that lack MBP, and their expression was carried out according to Sulistijo *et al*³⁸. Routinely the total protein concentration of lysate was determined using a colorimetric assay (Pierce). The spectrophotometric assay of Shaw⁶⁰

was used to detect chloramphenicol acetyltransferase activity in cell lysates. In this method the generation of coenzyme A upon transfer of an acetyl group from acetyl-CoA to chloramphenicol is quantified by the color change at 412 nm that occurs after reaction of the free thiol of CoASH with 5,5'-dithiobis-(2-nitrobenzoic acid). 32 μ l of lysate was mixed with 800 μ l of reaction buffer (0.1 mM Acetyl-CoA, 0.4 mg/ml 5,5'-dithiobis-(2-nitrobenzoic acid), 0.1 M Tris-HCl, pH 7.8), and the absorbance was acquired for 2 min to determine a background rate of acetyl-CoA hydrolysis. 32 μ l of 2.5mM Chloramphenicol was then added with mixing, and the absorbance was followed for 1 min. Each lysate was assayed in triplicate and the reported data are the averages of at least three different experiments. All kinetics measurements were performed at 37°C on a UV1 Thermoelectron corporation spectrophotometer.

Western Blots

2 ml aliquots of crude culture were centrifuged at 5000xg and the resultant pellet was resuspended in 1x SDS-PAGE sample loading buffer, heated to 95°C for 5 min. The samples were analyzed on pre-cast 12% polyacrylamide gels (Pierce), transferred to a polyvinylidene fluoride membrane (Millipore), detected with an anti-MBP primary antibody (1:10,000; New England Biolabs) and visualized with an anti-mouse horseradish peroxidase conjugated and ECL reagent (Pierce).

Maltose Complementation assays

E. coli NT326 cells expressing ToxR'(TM)MBP constructs were incubated overnight in liquid M9 minimal medium containing 0.4% glucose, washed, streaked on M9 minimal media plates containing 0.4% maltose as the only carbon source, and incubated for 2 days at 37 °C.

Sulfhydryl Group Determination

The number of accessible sulfhydryl groups in the cysteine protein variants APP- β CTF (S730C) and MBP-APP- β CTF (S421C) was determined spectrophotometrically using DTNB³⁹. The protein was first desalted to remove DTT. Subsequently, an excess of DTNB was added (10 μ l from a 10 mM stock in ethanol) to a 1mL aliquot of the variants. The number of sulfhydryl groups was calculated from the increase in absorbance at 412 nm using an $\epsilon_{412} = 13\,600\text{ cm}^{-1}\text{ M}^{-1}$

Visualization of APP- β CTF dimers by electron microscopy

The APP- β CTF cysteine variant (S730C) purified in DDM was labeled with monomaleimidonanogold (Nanoprobes, Yaphank, NY) according to the manufacturer instructions. Two microliters of this reaction mixture were applied onto a glow discharged carbon coated copper grid. The partially blotted grid was applied onto the surface of a 40 μ l droplet of gold developer reagent (Nanoprobes, Yaphank, NY) for 20 seconds. Gold enhancing is necessary to increase the diameter of the 1.4 nm gold particles for optimal visualization under negative stain⁶¹. Under this technique, gold is deposited in a controlled and specific fashion around the initial gold particle and the resulting 5-20 nm gold particles can be easily visualized under negative stain. The grid was then rinsed with deionized water and stained with and 1% uranyl acetate. The specimen was imaged with a JEOL-2100 transmission electron microscope and images were recorded on a Tietz 2kx2k CCD camera at a nominal magnification of 10,000x. At this magnification the effective pixel size at the specimen was 14.5Å. Particle selection and measurement were performed with the program ImageJ.

Fluorescence Labeling

The labeling reaction was started by the addition of a 100 μ L aliquot of the fluorescence probe IANBD (from a 2mM stock in acetonitrile) to the variants APP- β CTF-S730C and APP- β CTF-TMD-S421C, previously desalted to remove the DTT. The resulting mixture was incubated in the dark at room temperature for 1h. After this time, the reaction mixture was loaded onto a PD-10 size exclusion column Q-Sepharose column equilibrated in buffer (50 mM Tris-HCl, pH 7.5, and 0.1% DDM) and eluted according to the manufacturers instructions (GE). The labeling of the variants with IAEDANS was performed in an analogous manner. An identical procedure was also used to label APP- β CTF with Oregon green 488 maleimide as a donor and Texas red C2 maleimide as an acceptor. The labeling efficiency was determined spectrophotometrically and defined as the percentage of the label concentration over the protein concentration.

Fluorescence Resonance Energy Transfer Measurements

Unless indicated otherwise, all FRET experiments were performed by mixing the donor labeled protein in a 1 : 4 ratio with the unlabelled protein or with the acceptor labeled protein in buffer (1 μ M total protein, 50 mM Tris-HCl, pH 7.5, 300mM NaCl, and 0.1% DDM). A donor/acceptor ratio of 1 : 4 was used to maximize the fraction of protein dimers containing donor-acceptor pairs. Fluorescence intensity emission spectra were recorded using a PC1 photon counting spectrofluorometer (ISS Inc, USA) at room temperature, in a 0.5 mL cuvette. Both excitation and emission slit widths were 5 nm. The fluorescence spectra were recorded at an excitation wavelength of either 339 or 490 and with an emission wavelength range either 400-600 or 500-700 nm. Energy transfer E was calculated from the measured peak donor labeled APP- β CTF fluorescence intensity in the absence and presence of the acceptor labeled APP- β CTF according to

$$E = 1 - (F_{DA}/F_D) \quad (1)$$

where F_{DA} and F_D are donor intensities of donor/acceptor labeled APP- β CTF and donor labeled APP- β CTF, respectively. The energy transfer due to sequence-specific TMD dimerization E_D can be calculated by:

$$E_D = f_D P_D E_R \quad (2)$$

where f_D is the fraction of molecules in the dimeric state, P_D is the probability for donor quenching in the dimer, and E_R is the energy transfer in the dimer. If we approximate by considering that the distance between the FRET pairs is smaller than the Förster distance then $E_R \approx 1$. Moreover, since our acceptor to donor ratio in all experiments is 4 we can consider $P_D \approx 1$. Thus, we can simplify equation (2) as:

$$E_D \approx f_D \quad (3)$$

Thus, substituting for equation (1) and taking into account that $f_D = 2[D]/[T]$ - where $[D]$ is the dimer concentration and $[T]$ is the total APP- β CTF concentration - we can write

$$f_D = 1 - (F_{DA}/F_D) = 2[D]/[T] \quad (4)$$

Finally, since $[M] = [T] - 2[D]$ the dissociation constant K_d is given by

$$K_d = [M]^2 / [D] \quad (5)$$

The competition experiments were performed by titrating increasing amounts of unlabeled MBP-APP- β CTF (in a MBP-APP- β CTF : APP- β CTF molar ratio of 0.1, 0.15, 0.25, and 0.5) to a labeled 1 : 4 mixture of AEDANS-APP- β CTF : ANBD-APP- β CTF (0.4 μ M total APP- β CTF).

Molecular Modeling and Simulations

Four different structural models of non-interacting or interacting APP- β CTF TMD helices (residues 699-724), either in their WT or mutant forms, in right-crossing or left-crossing configurations, were used as starting points for MD simulations. One of the initial models of APP- β CTF TMD dimers was obtained by homology modeling using the NMR structure of the GpA dimer in DPPC micelles (PDB entry code: 1AFO) as a structural template. This model was built with Modeller 9v3⁶², aligning the ⁷⁰⁴GXXXG⁷⁰⁸ sequence of APP- β CTF TMD with the ⁷⁹GXXXG⁸³ motif of GpA. The rest of the models were built by steering initial “right-crossing” or left-crossing dimeric configurations of the APP- β CTF TMD dimer so as to separate the constituent helices from each other.

All initial model structures were CG using the description of the MARTINI force field⁶³. The accuracy of this computational scheme was recently assessed by Samson and coworkers by demonstrating that all-atom and CG simulations of a typical membrane protein model system, such as GpA, produce qualitatively similar results (see for example⁶⁴). In consideration of more accurate results obtained for this system⁶⁵, the backbone particle type of glycine residues was changed from N0 to Nm. The APP- β CTF TMD systems were then embedded, one at the time, in a pre-equilibrated DPPC bilayer membrane, using the inflation/deflation protocol described by Tieleman and colleagues⁶⁶. The simulated systems resulted in a 6.0×6.0×8.0 nm unit cell containing a total of approximately 3,000 particles. MD simulations were carried out in the NPT ensemble under periodic boundary conditions, using the Berendsen coupling scheme⁶⁷ with a barostat time constant of 4.0 ps to maintain a constant pressure of 1 bar, and using a velocity-rescaling coupling scheme with a time constant of 0.1 ps to maintain a constant temperature of 323 K. The Linear Constraint Solver (LINCS) algorithm⁶⁸ was used to preserve the bond lengths. Lennard-Jones interactions were treated with a twin-range cutoff of 0.9 nm and 1.2 nm and electrostatic interactions were shifted from 0 nm to 1.2 nm. An integration time step of 20 fs was used for all simulations.

All simulations were performed using GROMACS 4.0⁶⁹ augmented by the Plumed 1.2 package for the implementation of metadynamics⁷⁰. For the metadynamics part, the distance between the COMs of the two interacting helices and the crossing angle (calculated as the angle between the planes defined respectively by (i) the COM of the N-terminal half of helix 1 (residues 1-13), the C-terminal half of helix 1 (14-26) and the C-terminal of helix 2 and (ii) the C-terminal half of helix 2, the N-terminal half of helix 2 and the N-terminal half of helix 1) were chosen as collective variables. Gaussian hills were added at a rate of 0.1 kJ/mol each 20 ps, with widths of 0.05 nm for the distance and 0.05 radians (i.e. $\sim 3^\circ$) for the crossing angle. Diffusion of the distance and the crossing angle were monitored to ensure that, during consecutive depositions, these collective variables were thoroughly explored.

The well-tempered metadynamics algorithm⁷¹ was used for the choice of the hill height with a temperature bias of T=1300 K. To efficiently exploit parallel machines, we used the multiple walker approach⁷², in which several simulations explore the same free-energy

surface and interact by contributing to the same history-dependent bias potential. Four conformations generated as described above were used as starting points for simulations totaling 5 μ s, and using four replicas whose bias potential was updated every 1 ns. The height of the hills was monitored throughout the simulations and was found to drop below 0.01 kJ/mol, which guaranteed the convergence of the simulations. To characterize the structural variability within each basin, the structures corresponding to regions within a free-energy of 2 kcal/mol from the minimum were extracted from the trajectory and clustered using the total root mean square deviation (RMSD) as a dissimilarity matrix and a single linkage agglomerative algorithm (as implemented in the Gromacs utilities package)⁷³.

Since the metadynamics algorithm introduces an external bias to the free-energy, the population of states sampled during the simulation does not reflect the correct Boltzmann distribution. In order to assign a correct statistical weight to each conformation, we monitored the “azimuthal” angles α_1 and α_2 (see Figure 3). Specifically, α_1 was defined as the angle formed by the vector connecting the COMs of the two helices and the vector connecting the COM of helix 1 and the COMs of residues G770, G704, and G708 within helix 1. It thus represents which face of helix 1 is at the dimeric interface. Similarly, α_2 was defined as the vector connecting the COM of the two interacting helices, and the vector connecting the COM of helix 2 and the COMs of residues G770, G704, and G708 within helix 2. The relative probability of conformations displaying different interfaces within each mayor basin, was then calculated from the unbiased distribution obtained following the algorithm described in⁷⁴.

Representative frames from each cluster were converted back to all atom structures using the protocol proposed by Rzepiela and colleagues⁷⁵. Briefly, this method employs a simulated annealing MD simulation in which the CG and atomistic structures are coupled via restraints. The resulting all-atom structures were then embedded into a pre-equilibrated all atom membrane, and solvated in a similar fashion to that described above for the CG systems. These systems were energy minimized and equilibrated by performing cycles of MD under successively relaxed position restraints (1000 kJ/mol·nm², 100 kJ/mol·nm², 10 kJ/mol·nm² and 0 kJ/mol·nm²) for a total of 10 ps. A further 1 ns of unrestrained simulation was carried out and analysis of interprotomer contacts was performed on these systems.

Calculation of dimerization constants

Experimental concentrations were measured and are reported as number of moles in the total sample volume V_{Tot} . Using this scale, the apparent dimerization constant is $K_{\text{app}} = (n_{\text{Dim}}/V_{\text{Tot}})/(n_{\text{Mon}}/V_{\text{Tot}})^2$. In order to relate free-energies to a common reference state, it is convenient⁷⁶ to use the mole fraction scale to indicate concentrations, and define a mole fraction dimerization constant as $K_X = (n_{\text{Dim}}/n_{\text{Det}})/(n_{\text{Mon}}/n_{\text{Det}})^2$. This constant is related to the apparent constant by $K_X = K_{\text{app}} [\text{Det}]$, where $[\text{Det}]$ is the molar concentration of the detergent in the bulk solution. Thus, the mole fraction standard free-energy can be expressed as $\Delta G^\circ = -RT \ln(K_X) = -RT \ln(K_{\text{app}} [\text{Det}])$. Since the membrane is a two-dimensional medium, another natural scale for the concentrations is the number of moles per unit area. Using this scale, it is possible to calculate the value of the dimerization constant by integrating the one-dimensional free-energy profile $W(r)$ (see, for instance⁷⁷), and then converting the value to the mole fraction scale using the area per lipid molecule A/n (0.62 nm² for the DPPC model used in our calculations):

$$K_X = \frac{A}{n_{\text{Det}}} \times \frac{2\pi}{e^{-W(r_M)/k_B T}} \int_0^{r_D} r e^{-W(r)/k_B T} dr \quad (6)$$

where $r_M=1.9$ nm is the radius that defines the reference state of the non-interacting helices, and $r_D=1.2$ nm is the value that separates interacting from non-interacting helix configurations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AD	Alzheimer's disease
APH-1	anterior pharynx defective 1
APP	amyloid precursor protein
APP-βCTF	amyloid precursor protein's β -carboxyl-terminal fragment
Aβ	amyloid- β peptide
BACE1	β -site of APP cleaving enzyme 1
BCL2	B-cell lymphoma 2
CAT	chloramphenicol acetyltransferase
CG	coarse grained
COM	center of mass
DDM	dodecyl maltoside
DPPC	dipalmitoyl phosphatidylcholine
FRET	fluorescence resonance energy transfer
GpA	Glycophorin A
IAEDANS	5-({[(2-iodoacetyl)amino]ethyl}amino)naphthalene-1-sulfonic acid
IANBD	N,N-dimethyl-N-(iodoacetyl)-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine
LINCS	Linear Constraint Solver
MBP	Maltose Binding Protein
MBP-APP-βCTF	a chimera of MBP and the TMD of APP- β CTF
MD	molecular dynamics
Ni-NTA	nickel-nitrilotriacetic acid
PEN-2	presenilin enhancer 2
PMSF	phenylmethylsulfonyl fluoride
RMSD	root mean square deviation

SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
WT	wild-type
TMD	transmembrane domain

References

1. Price DL, Sisodia SS, Borchelt DR. Genetic neurodegenerative diseases: the human illness and transgenic models. *Science*. 1998; 282:1079–83. [PubMed: 9804539]
2. Glenner GG, Wong CW, Quaranta V, Eanes ED. The amyloid deposits in Alzheimer's disease: their nature and pathogenesis. *Appl Pathol*. 1984; 2:357–69. [PubMed: 6242724]
3. Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev*. 2001; 81:741–66. [PubMed: 11274343]
4. Sisodia SS, George-Hyslop PH. gamma-Secretase, Notch, Abeta and Alzheimer's disease: where do the presenilins fit in? *Nat Rev Neurosci*. 2002; 3:281–90. [PubMed: 11967558]
5. Edbauer D, Winkler E, Regula JT, Pesold B, Steiner H, Haass C. Reconstitution of gamma-secretase activity. *Nat Cell Biol*. 2003; 5:486–8. [PubMed: 12679784]
6. Weidemann A, Eggert S, Reinhard FB, Vogel M, Paliga K, Baier G, Masters CL, Beyreuther K, Evin G. A novel epsilon-cleavage within the transmembrane domain of the Alzheimer amyloid precursor protein demonstrates homology with Notch processing. *Biochemistry*. 2002; 41:2825–35. [PubMed: 11851430]
7. Zhao G, Cui MZ, Mao G, Dong Y, Tan J, Sun L, Xu X. gamma-Cleavage is dependent on zeta-cleavage during the proteolytic processing of amyloid precursor protein within its transmembrane domain. *J Biol Chem*. 2005; 280:37689–97. [PubMed: 16157587]
8. Price DL, Sisodia SS. Mutant genes in familial Alzheimer's disease and transgenic models. *Annu Rev Neurosci*. 1998; 21:479–505. [PubMed: 9530504]
9. Siman R, Reaume AG, Savage MJ, Trusko S, Lin YG, Scott RW, Flood DG. Presenilin-1 P264L knock-in mutation: differential effects on abeta production, amyloid deposition, and neuronal vulnerability. *J Neurosci*. 2000; 20:8717–26. [PubMed: 11102478]
10. Moehlmann T, Winkler E, Xia X, Edbauer D, Murrell J, Capell A, Kaether C, Zheng H, Ghetti B, Haass C, Steiner H. Presenilin-1 mutations of leucine 166 equally affect the generation of the Notch and APP intracellular domains independent of their effect on Abeta 42 production. *Proc Natl Acad Sci U S A*. 2002; 99:8025–30. [PubMed: 12048239]
11. Schroeter EH, Ilagan MX, Brunkan AL, Hecimovic S, Li YM, Xu M, Lewis HD, Saxena MT, De Strooper B, Coonrod A, Tomita T, Iwatsubo T, Moore CL, Goate A, Wolfe MS, Shearman M, Kopan R. A presenilin dimer at the core of the gamma-secretase enzyme: insights from parallel analysis of Notch 1 and APP proteolysis. *Proc Natl Acad Sci U S A*. 2003; 100:13075–80. [PubMed: 14566063]
12. Qi Y, Morishima-Kawashima M, Sato T, Mitsumori R, Ihara Y. Distinct mechanisms by mutant presenilin 1 and 2 leading to increased intracellular levels of amyloid beta-protein 42 in Chinese hamster ovary cells. *Biochemistry*. 2003; 42:1042–52. [PubMed: 12549925]
13. Walker ES, Martinez M, Brunkan AL, Goate A. Presenilin 2 familial Alzheimer's disease mutations result in partial loss of function and dramatic changes in Abeta 42/40 ratios. *J Neurochem*. 2005; 92:294–301. [PubMed: 15663477]
14. Kumar-Singh S, Theuns J, Van Broeck B, Pirici D, Vennekens K, Corsmit E, Cruts M, Dermaut B, Wang R, Van Broeckhoven C. Mean age-of-onset of familial Alzheimer disease caused by presenilin mutations correlates with both increased Abeta42 and decreased Abeta40. *Hum Mutat*. 2006; 27:686–95. [PubMed: 16752394]
15. Bentahir M, Nyabi O, Verhamme J, Tolia A, Horre K, Wiltfang J, Esselmann H, De Strooper B. Presenilin clinical mutations can affect gamma-secretase activity by different mechanisms. *J Neurochem*. 2006; 96:732–42. [PubMed: 16405513]

16. Shen J, Kelleher RJ 3rd. The presenilin hypothesis of Alzheimer's disease: evidence for a loss-of-function pathogenic mechanism. *Proc Natl Acad Sci U S A*. 2007; 104:403–9. [PubMed: 17197420]
17. Kaneko H, Kakita A, Kasuga K, Nozaki H, Ishikawa A, Miyashita A, Kuwano R, Ito G, Iwatsubo T, Takahashi H, Nishizawa M, Onodera O, Sisodia SS, Ikeuchi T. Enhanced accumulation of phosphorylated alpha-synuclein and elevated beta-amyloid 42/40 ratio caused by expression of the presenilin-1 deltaT440 mutant associated with familial Lewy body disease and variant Alzheimer's disease. *J Neurosci*. 2007; 27:13092–7. [PubMed: 18045903]
18. Lemmon MA, Flanagan JM, Treutlein HR, Zhang J, Engelman DM. Sequence specificity in the dimerization of transmembrane alpha-helices. *Biochemistry*. 1992; 31:12719–25. [PubMed: 1463743]
19. Brosig B, Langosch D. The dimerization motif of the glycophorin A transmembrane segment in membranes: importance of glycine residues. *Protein Sci*. 1998; 7:1052–6. [PubMed: 9568912]
20. Russ W. The GxxxG motif: A framework for transmembrane helix-helix association. *Journal of Molecular Biology*. 2000; 296:911–919. [PubMed: 10677291]
21. Langosch D, Heringa J. Interaction of transmembrane helices by a knobs-into-holes packing characteristic of soluble coiled coils. *Proteins*. 1998; 31:150–9. [PubMed: 9593189]
22. Zhou FX, Cocco MJ, Russ WP, Brunger AT, Engelman DM. Interhelical hydrogen bonding drives strong interactions in membrane proteins [see comments]. *Nat Struct Biol*. 2000; 7:154–60. [PubMed: 10655619]
23. Zhou FX, Merianos HJ, Brunger AT, Engelman DM. Polar residues drive association of polyleucine transmembrane helices. *Proc Natl Acad Sci U S A*. 2001; 98:2250–5. [PubMed: 11226225]
24. McClain MS, Iwamoto H, Cao P, Vinion-Dubiel AD, Li Y, Szabo G, Shao Z, Cover TL. Essential role of a GXXXG motif for membrane channel formation by *Helicobacter pylori* vacuolating toxin. *J Biol Chem*. 2003; 278:12101–8. [PubMed: 12562777]
25. Mendrola JM, Berger MB, King MC, Lemmon MA. The single transmembrane domains of ErbB receptors self-associate in cell membranes. *J Biol Chem*. 2002; 277:4704–12. [PubMed: 11741943]
26. MacKenzie KR, Prestegard JH, Engelman DM. A transmembrane helix dimer: structure and implications. *Science*. 1997; 276:131–3. [PubMed: 9082985]
27. Senes A, Ubarretxena-Belandia I, Engelman DM. The Calpha ---H...O hydrogen bond: a determinant of stability and specificity in transmembrane helix interactions. *Proc Natl Acad Sci U S A*. 2001; 98:9056–61. [PubMed: 11481472]
28. Vooijs M. Ectodomain Shedding and Intramembrane Cleavage of Mammalian Notch Proteins Is Not Regulated through Oligomerization. *Journal of Biological Chemistry*. 2004; 279:50864–50873. [PubMed: 15448134]
29. Munter L-M, Voigt P, Harmeier A, Kaden D, Gottschalk KE, Weise C, Pipkorn R, Schaefer M, Langosch D, Multhaup G. GxxxG motifs within the amyloid precursor protein transmembrane sequence are critical for the etiology of A 42. *EMBO J*. 2007; 26:1702–1712. [PubMed: 17332749]
30. Kienlen-Campard P, Tasiaux B, Van Hees J, Li M, Huysseune S, Sato T, Fei JZ, Aimoto S, Courttoy PJ, Smith SO, Constantinescu SN, Octave J-N. Amyloidogenic processing but not amyloid precursor protein (APP) intracellular C-terminal domain production requires a precisely oriented APP dimer assembled by transmembrane GXXXG motifs. *J Biol Chem*. 2008; 283:7733–44. [PubMed: 18201969]
31. Gorman PM, Kim S, Guo M, Melnyk RA, McLaurin J, Fraser PE, Bowie JU, Chakrabarty A. Dimerization of the transmembrane domain of amyloid precursor proteins and familial Alzheimer's disease mutants. *BMC Neurosci*. 2008; 9:17. [PubMed: 18234110]
32. Sato T, Tang T, Reubins G, Fei J, Fujimoto T, Kienlen-Campard P, Constantinescu S, Octave J, Aimoto S, Smith S. A helix-to-coil transition at the {varepsilon}-cut site in the transmembrane dimer of the amyloid precursor protein is required for proteolysis. *Proc Natl Acad Sci USA*. 2009
33. Russ WP, Engelman DM. TOXCAT: a measure of transmembrane helix association in a biological membrane. *Proc Natl Acad Sci U S A*. 1999; 96:863–8. [PubMed: 9927659]

34. Li YM, Lai MT, Xu M, Huang Q, DiMuzio-Mower J, Sardana MK, Shi XP, Yin KC, Shafer JA, Gardell SJ. Presenilin 1 is linked with gamma-secretase activity in the detergent solubilized state. *Proc Natl Acad Sci U S A*. 2000; 97:6138–43. [PubMed: 10801983]
35. Fraering PC, Ye W, Strub J-M, Dolios G, LaVoie MJ, Ostaszewski BL, van Dorsselaer A, Wang R, Selkoe DJ, Wolfe MS. Purification and characterization of the human gamma-secretase complex. *Biochemistry*. 2004; 43:9774–89. [PubMed: 15274632]
36. Esler WP, Kimberly WT, Ostaszewski BL, Ye W, Diehl TS, Selkoe DJ, Wolfe MS. Activity-dependent isolation of the presenilin- gamma -secretase complex reveals nicastrin and a gamma substrate. *Proc Natl Acad Sci U S A*. 2002; 99:2720–5. [PubMed: 11867728]
37. Arkin IT, Adams PD, MacKenzie KR, Lemmon MA, Brunger AT, Engelman DM. Structural organization of the pentameric transmembrane alpha-helices of phospholamban, a cardiac ion channel. *Embo J*. 1994; 13:4757–64. [PubMed: 7525269]
38. Sulistijo ES. Sequence-specific Dimerization of the Transmembrane Domain of the “BH3-only” Protein BNIP3 in Membranes and Detergent. *Journal of Biological Chemistry*. 2003; 278:51950–51956. [PubMed: 14532263]
39. Ellman GL. Tissue Sulfhydryl Groups. *Arch. Biochem. Biophys*. 1959; 82:70–77. [PubMed: 13650640]
40. Ubarretxena-Belandia I, Hozeman L, van der Brink-van der Laan E, Pap EH, Egmond MR, Verheij HM, Dekker N. Outer membrane phospholipase A is dimeric in phospholipid bilayers: a cross-linking and fluorescence resonance energy transfer study. *Biochemistry*. 1999; 38:7398–405. [PubMed: 10353852]
41. Selvin PR. Fluorescence resonance energy transfer. *Methods Enzymol*. 1995; 246:300–34. [PubMed: 7752929]
42. Franzen JS, Marchetti PS, Feingold DS. Resonance energy transfer between catalytic sites of bovine liver uridine diphosphoglucose dehydrogenase. *Biochemistry*. 1980; 19:6080–6089. [PubMed: 7470452]
43. Dawson J. Sequence Context Strongly Modulates Association of Polar Residues in Transmembrane Helices. *Journal of Molecular Biology*. 2003; 331:255–262. [PubMed: 12875850]
44. Li R. Dimerization of the Transmembrane Domain of Integrin IIb Subunit in Cell Membranes. *Journal of Biological Chemistry*. 2004; 279:26666–26673. [PubMed: 15067009]
45. Constantinescu SN. The Erythropoietin Receptor Transmembrane Domain Mediates Complex Formation with Viral Anemic and Polycythemic gp55 Proteins. *Journal of Biological Chemistry*. 2003; 278:43755–43763. [PubMed: 12930840]
46. Chin C, Sachs J, Engelman D. Transmembrane homodimerization of receptor-like protein tyrosine phosphatases. *FEBS Letters*. 2005; 579:3855–3858. [PubMed: 15978577]
47. Landolt-Marticorena C, Williams KA, Deber CM, Reitchmeier RAF. Non-random distribution of amino acids in the transmembrane segments of human type I single span membrane proteins. *J Mol Biol*. 1993; 229:602–608. [PubMed: 8433362]
48. Arkin IT, Brunger AT. Statistical analysis of predicted transmembrane alpha-helices. *Biochim Biophys Acta*. 1998; 1429:113–28. [PubMed: 9920390]
49. Kumar-Singh S, De Jonghe C, Cruys M, Kleinert R, Wang R, Mercken M, De Strooper B, Vanderstichele H, Lofgren A, Vanderhoeven I, Backhovens H, Vanmechelen E, Kroisel PM, Van Broeckhoven C. Nonfibrillar diffuse amyloid deposition due to a gamma(42)-secretase site mutation points to an essential role for N-truncated A beta(42) in Alzheimer's disease. *Hum Mol Genet*. 2000; 9:2589–98. [PubMed: 11063718]
50. Carter DA, Desmarais E, Bellis M, Campion D, Clerget-Darpoux F, Brice A, Agid Y, Jaillard-Serradt A, Mallet J. More missense in amyloid gene. *Nat Genet*. 1992; 2:255–6. [PubMed: 1303275]
51. Pasalar P, Najmabadi H, Noorian AR, Moghimi B, Jannati A, Soltanzadeh A, Krefft T, Crook R, Hardy J. An Iranian family with Alzheimer's disease caused by a novel APP mutation (Thr714Ala). *Neurology*. 2002; 58:1574–5. [PubMed: 12034808]
52. Murrell J, Farlow M, Ghetti B, Benson MD. A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science*. 1991; 254:97–9. [PubMed: 1925564]

53. Murrell JR, Hake AM, Quaid KA, Farlow MR, Ghetti B. Early-onset Alzheimer disease caused by a new mutation (V717L) in the amyloid precursor protein gene. *Arch Neurol.* 2000; 57:885–7. [PubMed: 10867787]
54. Chartier-Harlin MC, Crawford F, Houlden H, Warren A, Hughes D, Fidani L, Goate A, Rossor M, Roques P, Hardy J, et al. Early-onset Alzheimer's disease caused by mutations at codon 717 of the beta-amyloid precursor protein gene. *Nature.* 1991; 353:844–6. [PubMed: 1944558]
55. Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, et al. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature.* 1991; 349:704–6. [PubMed: 1671712]
56. Langosch D, Brosig B, Kolmar H, Fritz HJ. Dimerisation of the glycophorin A transmembrane segment in membranes probed with the ToxR transcription activator. *J Mol Biol.* 1996; 263:525–30. [PubMed: 8918935]
57. Adams PD, Engelman DM, Brunger AT. Improved prediction for the structure of the dimeric transmembrane domain of glycophorin A obtained through global searching [published erratum appears in *Proteins* 1997 Jan;27(1):132]. *Proteins.* 1996; 26:257–61. [PubMed: 8953647]
58. Miyashita N, Straub JE, Thirumalai D, Sugita Y. Transmembrane structures of amyloid precursor protein dimer predicted by replica-exchange molecular dynamics simulations. *J Am Chem Soc.* 2009; 131:3438–9. [PubMed: 19275251]
59. Zhang J, Lazaridis T. Calculating the free energy of association of transmembrane helices. *Biophys J.* 2006; 91:1710–23. [PubMed: 16766613]
60. Shaw WV. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. *Methods Enzymol.* 1975; 43:737–755. [PubMed: 1094240]
61. Weipoltshammer K, Schofer C, Almeder M, Wachtler F. Signal enhancement at the electron microscopic level using Nanogold and gold-based autometallography. *Histochem Cell Biol.* 2000; 114:489–95. [PubMed: 11201611]
62. Sali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol.* 1993; 234:779–815. [PubMed: 8254673]
63. Marrink SJ, Risselada HJ, Yefimov S, Tieleman DP, de Vries AH. The MARTINI force field: coarse grained model for biomolecular simulations. *J Phys Chem B.* 2007; 111:7812–24. [PubMed: 17569554]
64. Psachoulia E, Marshall DP, Sansom MS. Molecular dynamics simulations of the dimerization of transmembrane alpha-helices. *Acc Chem Res.* 2010; 43:388–96. [PubMed: 20017540]
65. Psachoulia E, Fowler PW, Bond PJ, Sansom MSP. Helix-helix interactions in membrane proteins: coarse-grained simulations of glycophorin a helix dimerization. *Biochemistry.* 2008; 47:10503–12. [PubMed: 18783247]
66. Kandt C, Ash WL, Tieleman DP. Setting up and running molecular dynamics simulations of membrane proteins. *Methods.* 2007; 41:475–88. [PubMed: 17367719]
67. Berendsen HJC, Postma JPM, van Gunsteren WF, A. D, Haak JR. Molecular dynamics with coupling to an external bath. *J.Chem. Phys.* 1984; 81:3684–3690.
68. Hess B, Bekker H, Berendsen HJC, Fraaije JGEM. LINCS: A linear constraint solver for molecular simulations. *Journal of Computational Chemistry.* 1997; 18:1463–1472.
69. Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJ. GROMACS: fast, flexible, and free. *J Comput Chem.* 2005; 26:1701–18. [PubMed: 16211538]
70. Bonomi M, Branduardi D, Bussi G, Camilloni C, Provasi D, Raiteri P, Donadio D, Marinelli F, Pietrucci F, Broglia RA, Parrinello M. PLUMED: A portable plugin for free-energy calculations with molecular dynamics. *Computer Physics Communications.* 2009; 180:1961–1972.
71. Barducci A, Bussi G, Parrinello M. Well-tempered metadynamics: a smoothly converging and tunable free-energy method. *Phys Rev Lett.* 2008; 100:020603. [PubMed: 18232845]
72. Raiteri P, Laio A, Gervasio FL, Micheletti C, Parrinello M. Efficient reconstruction of complex free energy landscapes by multiple walkers metadynamics. *J Phys Chem B.* 2006; 110:3533–9. [PubMed: 16494409]
73. Hess B, Kutzner C, van der Spoel D, Lindahl E. GROMACS 4: Algorithms for highly efficient, load-balanced, and scalable molecular simulation. *Journal of Chemical Theory and Computation.* 2008; 4:435–447.

74. Bonomi M, Barducci A, Parrinello M. Reconstructing the equilibrium Boltzmann distribution from well-tempered metadynamics. *J. Comput. Chem.* 2009; 30:1615–1621. [PubMed: 19421997]
75. Rzepiela AJ, Schafer LV, Goga N, Risselada HJ, De Vries AH, Marrink SJ. Reconstruction of atomistic details from coarse-grained structures. *J Comput Chem.* 2010; 31:1333–43. [PubMed: 20087907]
76. Fleming KG. Standardizing the free energy change of transmembrane helix-helix interactions. *J Mol Biol.* 2002; 323:563–71. [PubMed: 12381309]
77. Henin J, Pohorille A, Chipot C. Insights into the recognition and association of transmembrane alpha-helices. The free energy of alpha-helix dimerization in glycophorin A. *J Am Chem Soc.* 2005; 127:8478–84. [PubMed: 15941282]

Figure 1.

APP-βCTF is a transmembrane domain mediated dimer. **A.** Sequence of recombinant APP-βCTF (Top). The TMD is in bold, the Serine residue that was mutated to Cysteine is in red, and the C-terminal Flag-tag is underlined. The different cleavage sites by γ-secretase are marked. The residue numbering corresponds to the APP770 isoform. Sequence detail of the MBP-APP-βCTF chimera (bottom). A thrombin cleavage site separates MBP from the amino acid sequence corresponding to the TMD region of APP-βCTF (bold) and the C-terminus includes a His₆-tag (H6) for purification. The Serine residue that was mutated to Cysteine is in red. **B.** SDS-PAGE analysis of purified APP-βCTF. The molecular weight marker positions are shown on the left, and M and D denote the monomer and dimeric forms, respectively. Anti-Flag western blotting and mass spectrometry determined the identity of the bands corresponding to the monomeric and dimeric species (not shown). **C.** The fluorescence intensity emission spectrum of APP-βCTF in 0.1% DDM. The spectra of a 1 : 4 mixture of AEDANS-APP-βCTF : APP-βCTF (1), AEDANS-APP-βCTF : ANBD-APP-βCTF (2) and APP-βCTF : ANBD-APP-βCTF (3). **D.** Determination of the dimerization affinity of a 1 : 4 mixture of AEDANS-APP-βCTF : ANBD-APP-βCTF (black curve) and of AEDANS-MBP-APP-βCTF : ANBD-MBP-APP-βCTF (grey curve). The fluorescence intensity was measured as a function of total protein concentration. The fraction of dimer (f_D) was determined as described in materials and methods. **E.** Competition experiment performed by titrating increasing amounts of unlabeled MBP-APP-βCTF (in a MBP-APP-βCTF : APP-βCTF molar ratio of 0.1, 0.15, 0.25, and 0.5) to a labeled 1 : 4 mixture of AEDANS-APP-βCTF : ANBD-APP-βCTF (0.4 μM total APP-βCTF).

Figure 2.

Homo-oligomerization of the TMD of APP- β CTF as measured by the TOXCAT assay. **A.** Sequences of the TMD region of TOXCAT chimeras. Shown are the TMD regions of GpA WT (GpA WT) and the GpA G83I mutant (TMD residues L75-T87), and the TMD region of APP- β CTF (APP- β CTF WT; TMD residues K699-M722). The TMD sequences (capital letters) were inserted between the ToxR transcriptional activation domain (*ToxR*) and the MBP domain. Sequences that flank the inserted TMDs are shown in lowercase letters. **B.** Cells expressing the TOXCAT chimeras were lysed and assayed for CAT activity. **C.** Cells expressing the TOXCAT chimeras carrying single phenylalanine substitutions along the TMD region of APP- β CTF were lysed and assayed for CAT activity.

Figure 3.

Collective variables for the metadynamics simulations depicted on a APP- β CTF TMD dimeric model. The locations of the centers of mass of the N-terminal halves (respectively in light blue and light purple) and of the C-terminal halves (in dark blue and dark purple) are indicated with gray spheres.

Figure 4.

Two-dimensional free-energy surface of the WT sequence. The location of the main basins A ($-30^\circ < \Omega < -20^\circ$; $d = 7.2 \text{ \AA}$) and B ($-10^\circ < \Omega < +5^\circ$; $d = 8.2 \text{ \AA}$) is indicated with labels. Each contour represents a free-energy difference of 1 kcal/mol.

Figure 5.
Representative configurations of clusters deriving from the WT simulation of APP- β CTF TMD.

Figure 6. Mono-dimensional free-energy curves $G(d)$ for WT (black curves) and mutant APP- β CTF TMD. All free-energies are shifted to a common reference value for the region $d > 1.9$ nm, corresponding to non-interacting helices. The right panel reports the curves for the mutants with no effect on the CAT expression, and the left panel the mutants with significantly reduced CAT expression.

Table 1

Free-energy difference between the monomeric reference state ($d=1.9$ nm) and the dimeric state are reported in the first column for the WT system and selected mutants. The third column reports the variation of dimeric stability upon mutation.

Sequence	ΔG [kcal/mol]	$\Delta\Delta G$ [kcal/mol]
WT	-9.5	-
G700F	-6.5	+3.0
V701F	-7.0	+2.5
I702F	-7.0	+2.5
I703F	-7.0	+2.5
G709F	-9.0	+0.5
A713F	-9.0	+0.5
V715F	-6.5	+3.0
V717F	-9.0	+0.5
V717G	-9.2	+0.3
V717I	-8.8	+0.7
V717L	-9.0	+0.5