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Familial Alzheimer's mutations within APPTM increase A β 42 production by enhancing accessibility of ϵ -cleavage site

Wen Chen¹, Eric Gamache¹, David J. Rosenman¹, Jian Xie¹, Maria M. Lopez¹, Yue-Ming Li² & Chunyu Wang¹

The high A β 42/A β 40 production ratio is a hallmark of familial Alzheimer's disease, which can be caused by mutations in the amyloid precursor protein (APP). The C-terminus of A β is generated by γ -secretase cleavage within the transmembrane domain of APP (APPTM), a process that is primed by an initial ϵ -cleavage at either T48 or L49, resulting in subsequent production of A β 42 or A β 40, respectively. Here we solve the dimer structures of wild-type APPTM (AAPT_{WT}) and mutant APPTM (FAD mutants V44M) with solution NMR. The right-handed APPTM helical dimer is mediated by GXXXA motif. From the NMR structural and dynamic data, we show that the V44M and V44A mutations can selectively expose the T48 site by weakening helical hydrogen bonds and increasing hydrogen-deuterium exchange rate (k_{ex}). We propose a structural model in which FAD mutations (V44M and V44A) can open the T48 site γ -secretase for the initial ϵ -cleavage, and consequently shift cleavage preference towards A β 42.

¹Department of Biological Sciences, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 8th Street, Troy, New York 12180, USA. ²Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, New York 10065, USA. Correspondence and requests for materials should be addressed to C.W. (email: wangc5@rpi.edu).

Amyloid β -peptide (A β) is the major component of senile plaques, a pathologic hallmark of Alzheimer's disease (AD)¹. Amyloid precursor protein (APP) is an integral membrane protein with a single transmembrane (TM) domain, which is consecutively cleaved by β - and γ -secretase to generate amyloid beta (A β) peptides². A β 40 and A β 42, consisting of 40 and 42 residues, respectively, are the two major isoforms of A β in human brains^{1,3}. While A β 40 is relatively benign, A β 42 aggregates rapidly into neurotoxic oligomers and fibrils (plaques)⁴. A β 42 is not only toxic in neuronal culture⁵, but also in animal models⁶. Familial Alzheimer's disease (FAD) is caused by dominant mutations in genes encoding APP and γ -secretase. FAD is biochemically characterized by the increased ratio of A β 42 over A β 40 (refs 7,8), implicating the crucial role of A β 42 in AD pathogenesis. However, the mechanism of how FAD mutations increase A β 42/A β 40 remains poorly understood.

The proteolytic cleavage of APP is initiated by β -secretase within the extracellular domain of APP, releasing a C-terminal membrane-anchored fragment of 99 residues (C99)^{9,10}. C99 is further processed by γ -secretase within the TM domain of APP (APPTM) to yield mature A β peptides and the APP intracellular domain (AICD)^{11–13}. However, the cleavage of APPTM by γ -secretase is not specific, as varying lengths of A β peptides have been observed¹¹. A β 40 is the predominant product, while A β 42 is generated to a lesser extent¹. Another cleavage site, called the ϵ -cleavage site, has recently been established as the initial C99 cleavage site by γ -secretase, which generates A β 48 or A β 49 and AICD49–99 or AICD50–99, respectively¹⁴. Successive proteolysis by the processive carboxypeptidase-like activity of γ -secretase releases tripeptides and tetrapeptides from the C-terminus of A β 48 and A β 49. Two production lines are distinguished, one for A β 40 and the other for A β 42. A β 46, A β 43 and A β 40 are generated from A β 49, while A β 45, A β 42 and A β 38 are produced from A β 48 (ref. 14). Therefore, initial ϵ -cleavage at residue 48 (T48, γ -secretase cleaving between residues 48 and 49) or 49 (L49, cleaving between 49 and 50) determines whether A β 42 or A β 40, respectively, can be produced and can critically influence the A β 42/A β 40 ratio.

Twelve FAD mutations have been identified within the APPTM^{7,15–20}, which contains the ϵ - and γ -cleavage sites. These FAD mutations contribute to an increased A β 42/A β 40 ratio^{8,21}. Using APPTM as the substrate, we have developed a novel γ -secretase assay which reproduces A β 42/A β 40 ratios for WT APPTM and FAD mutants²². This suggests that APPTM itself, as a substrate for γ -secretase, mimics the behaviour of the natural substrate C99 and that APPTM plays an important role in determining A β 42/A β 40 ratios.

Although ample structural work on APPTM has been published, it remains unclear how FAD mutation within APPTM increase the A β 42/A β 40 ratio. Sanders' group presented the first solution NMR studies of C99 (refs 23,24), which includes APPTM, and a recent monomer structure relevant for cholesterol binding²³. However, the existence of APPTM as a dimer has been supported by numerous studies^{24–28}. Sato *et al.*²⁷ derived a dimeric structural model for APPTM based on limited constraints from solid-state NMR. Solid-state NMR also revealed both helical and non-helical conformations at the two termini and in the vicinity of the γ -secretase site in APPTM in lipids²⁹. Computational models of APPTM dimers were published by Miyashita *et al.*³⁰ and more recently by Wang *et al.*³¹ Finally, Nadezhddin *et al.*²⁸ reported the solution NMR structure of APPTM WT as a left-handed dimer, though this model is based on intermolecular NOEs from filtered experiments only and has not been refined with residual dipolar couplings (RDCs).

In this study, to explore the structural mechanism of increased A β 42/A β 40 ratio in FAD mutations within APPTM, we use

solution NMR spectroscopy to investigate the structure and dynamics of three variants of APPTM, wild type (WT) and two FAD mutants, V44M (French mutation) and V44A (German mutation). Because we have shown APPTM itself is an excellent substrate of γ -secretase and FAD mutations within APPTM produce similar effects on 42/40 ratios as in assays using C99 as the substrate²², our NMR studies of APPTM itself can be directly correlated to the specificity of γ -secretase cleavage and A β 42/A β 40 ratios. Here, we show that V44M and V44A significantly alter the structure and dynamics of T48, the ϵ -cleavage site for A β 42 production and propose a novel mechanism for increased A β 42/A β 40 ratio in FAD.

Results

V44M and V44A cause large CSP at the ϵ -cleavage site T48.

Isotopically labelled APPTM WT V44M were reconstituted in dodecylphosphocholine (DPC) micelles as described in Chen *et al.*³² and their complete assignments have been achieved with triple resonance experiments and deposited in BioMagResBank (BMRB entry 18,648 and 18,649). The residues are numbered according to A β (Supplementary Fig. S1). Figure 1a shows the ¹⁵N–¹H HSQC spectra of WT and V44M APPTM, with narrow dispersion typical of TM helices. The two spectra have similar peak patterns, suggesting that the V44M mutation does not change the overall fold of APPTM. Large chemical shift perturbations (CSPs) are observed at and near the site of the mutation (residues 44–48), as expected (Fig. 1b). While the largest ¹⁵N CSP is at V44M, the largest ¹H CSP occurs at T48 (Fig. 1b), whose amide proton chemical shift decreases from 8.14 p.p.m. in WT to 7.93 p.p.m. in the mutant. The ~0.2 p.p.m. CSP indicates significant changes in magnetic environment of T48 amide proton, most likely due to changes in hydrogen bonding. We also have compared the backbone amide chemical shifts of V44A, another FAD mutant, with WT (Fig. 1b). While the largest ¹⁵N CSP is at the site of mutation V44A, the largest ¹H CSP again occurs at T48 (Fig. 1b). As T48 is the initial recognition site of γ -secretase for the A β 42 production line (Supplementary Fig. S1)¹⁴, such changes in the local conformations of the ϵ -cleavage site may have important implications for the A β 42/A β 40 ratio.

V44M changes local conformations of ϵ -cleavage sites.

To further investigate the detailed structural changes brought about by the V44M mutation, we solved the solution structures of both APPTM WT and V44M dimers, which were based on unambiguous intermolecular NOEs (Fig. 2) and RDCs from two different alignment media (Table 1). The dimerization of APPTM in DPC micelles was confirmed by analytical ultracentrifugation (Supplementary Fig. S2) and supported by numerous previous studies^{24–28}. Unambiguous intermolecular NOEs were determined by a number of chimera NMR samples with selective isotopical labelling (Fig. 2). Because only one set of NMR resonances are observed (Fig. 1a), the structure was calculated as a symmetric dimer. On average, 25.4 (WT) and 25.9 (V44M) constraints per residue were obtained, including 9 intermolecular NOEs for WT and 11 intermolecular NOEs for V44M. An ensemble of 20 structures were calculated from XPLOR-NIH for WT and V44M (for stereo images of superimposed NMR structures, see Supplementary Figs S3 and S4), with backbone and heavy atom pairwise RMSDs at 0.79 and 1.28 Å (WT) and 0.81 and 1.31 Å (V44M), respectively (Table 1). The coordinates and constraints have been deposited in the protein data bank as 2LZ3 (WT) and 2LZ4 (V44M).

Both WT and V44M form right-handed dimers, as in the classical TM dimer of glycophorin A (GpA) (Fig. 3)³³. The crossing angle between the two helices is 22° for WT and 33° for

Figure 1 | FAD mutations within APPTM of V44M and V44A cause large changes in amide proton chemical shift of T48. (a) ^{15}N -H HSQC of APPTM WT (blue peaks), overlaid with that of V44M (red peaks). Residues M-2 and A-1 are non-APPTM residues N-terminal to APPTM due to cloning. (b) CSP caused by V44M (▲ joined by solid lines) and V44A (● joined by dashed lines) in amide proton (red) and nitrogen (black). Residues are numbered according to A β for easy correlation with A β production (Supplementary Fig. S1). In both FAD mutants, largest proton CSP occurs at T48. While the proton CSP pattern is similar to V44M, V44A causes larger nitrogen CSP at residues 44–46. Average backbone nitrogen chemical shifts of V, M, A are 121.08, 120.07 and 123.22 p.p.m., respectively, which mirrors the larger and opposite change in ^{15}N CSP at residue 44 in V44A compared with V44M.

V44M. For both APPTM WT and V44M, the GXXXX motif (residues 38–42) mediates the dimer interface (Fig. 3a). In contrast, the GXXXX motifs in APPTM do not have a direct role in dimerization. In addition, the side chains of M35, V39, I45, V46 and L49 provide key hydrophobic interactions at the dimer interface for the WT molecule. There is no hydrogen bond or salt bridge across the dimer interface.

The dimer fold and interface are generally retained in the V44M mutant but the dimerization pattern is altered significantly (Fig. 3b). The GXXXX motif and the side chains of M35, V39 and V46 still mediate dimerization; however, there is a registry shift in hydrophobic side-chain packing towards the C-terminus. I45 and L49 are no longer present at the dimer interface in V44M while T43 and V50 provide new hydrophobic packing for V44M dimerization. While T48 is not at the dimer interface in either structure, V44M mutation relocates L49 away from the dimer interface.

The large CSP in the T48 amide proton led us to more closely examine the helical hydrogen bonds. Overall, the V44M mutation strengthens hydrogen bonding at the N-terminal half of the helix while weakening those at the C-terminal half (Fig. 4). The helical hydrogen bonds involving the ϵ -cleavage site T48 and L49 amides experience large changes. In the 20 NMR structures, the average distance between the T48 amide proton and the V44 carbonyl oxygen is 2.2 ± 0.2 Å and 3.23 ± 0.14 Å in the WT and V44M mutant, respectively. The average distance between the L49 amide proton and the I45 carbonyl oxygen is 2.3 ± 0.2 Å and 2.8 ± 0.4 Å in WT and V44M, respectively. Thus the helical hydrogen bond involving both T48 and L49 amide protons are both weakened and the effect is much more pronounced for T48 than for L49.

V44M and V44A enhance k_{ex} of T48 more than L49. To further probe helical stability and dynamics with residue-specific resolution, we have carried out hydrogen–deuterium (HD) exchange measurements at 298 K (Fig. 5a). Helical core residues between I41 and I47 had no decrease in their signal intensity in HSQC spectra after 30 h in D_2O , due to very slow exchange with water, in both WT and V44M (Fig. 5a and Supplementary Fig. S5). Residues V36–V40 display decreasing exchange rates towards the centre of the helix while exchange rates of residues T48–V50 increase towards the C-terminus in both WT and V44M. T48 has an ~ 4 -fold enhancement in HD exchange rate, increasing from 0.009 per h in WT to 0.034 per h in V44M (Figs 4 and 5a), while L49 has an ~ 2 -fold increase (0.028 per h in WT versus 0.05 per h in V44M) (Fig. 5a). Consequently, both initial ϵ -cleavage sites are destabilized by V44M, but the effect is more significant for T48 than for L49. Titration with paramagnetic relaxation enhancement (PRE) probes demonstrated similar micelle embedment around the ϵ -cleavage sites in WT and V44M (Supplementary Fig. S6), suggesting the observed changes in HD exchange rates are not due to the differences in micelle embedment.

HD exchange experiment was also carried out for V44A, another FAD mutant. In V44A, T48 has a 4 fold enhancement in k_{ex} , increasing from 0.009 per h in WT to 0.036 per h in V44A (Fig. 5a), while L49 has only an $\sim 30\%$ fold increase (0.028 per h in WT versus 0.036 per h in V44M) (Fig. 5a). Such changes in k_{ex} may indicate enhanced accessibility of T48 site to ϵ -cleavage by γ -secretase. Indeed, it has been reported recently that V44A dramatically increase the ratio of AICD49-99/AICD50-99 (ref. 34), where AICD49-99 and AICD50-99 are the product of ϵ -cleavage at L48 and T49, respectively.

Discussion

For the first time, we have compared the structure and dynamics of WT APPTM and one of its FAD mutants V44M at atomic

Figure 2 | Unambiguous determination of intermolecular NOEs. Reciprocal intermolecular NOE peaks between V39HN and V39HG1 (a), and between V46HN and V46HG2 (b), detected in a 1:1 mixed sample of uniformly ^{13}C labelled APPTM and uniformly ^{15}N labelled APPTM, yielding unambiguous intermolecular NOEs between ^{13}C -H and ^{15}N -H. The left panel is the strip from ^{13}C -selected ^{15}N -NOESY, while the right panel is the strip from ^{15}N -selected ^{13}C -NOESY. (c) V40HN-V39G1 intermolecular NOE detected in a 1:1 mixed sample of uniformly ^{15}N -labelled and perdeuterated V44M, and natural abundance V44M, by 3D ^{15}N -NOESY, giving rise to the intermolecular NOEs between ^{15}N -H and aliphatic protons. (d) Intermolecular NOEs detected by filtered ^{13}C -NOESY in a 1:1 mixed sample of ^{13}C , ^{15}N -APPTM and unlabelled APPTM. A new intermolecular NOEs M44E-V49G1 appears in the V44M mutant.

resolution. We find that V44M mutation destabilizes the local helical conformation and increases dynamics at the ε -cleavage sites. These changes are especially pronounced for T48, the initial ε -cleavage site for the A β 42 production, as shown by the large amide proton CSP, lengthening of the helical hydrogen bonds and significantly amplified k_{ex} . Similar changes in CSP and HD exchange rate were obtained for V44A, another FAD mutant. These data indicate that T48 is much more prone to undergo helical unwinding in V44M and V44A than WT, suggesting a structural mechanism of how V44M and V44A can increase A β 42/A β 40 ratio (Fig. 5b). γ -Secretase first cleaves APPTM endoproteolytically at the ε -sites, at residues T48 or L49 (refs 14,35,36), followed by carboxypeptidase-like activity, resulting in two lines of A β production: the A β 42 line (48 $\rightarrow$ 45 $\rightarrow$ 42 $\rightarrow$ 38), and A β 40 line (49 $\rightarrow$ 46 $\rightarrow$ 43 $\rightarrow$ 40) (Supplementary Fig. S1). For the initial ε -cleavage to occur, the ε -site scissile bonds need to be accessible to the γ -secretase active site and catalytic water, which requires the unraveling of the local helical conformation. Because the T48 and L49 amides are adjacent to the scissile bond carbonyl within the same residue, these amides serve as excellent reporter for the accessibility of the ε -sites. While the V44M and V44A mutations destabilize the local helical conformation of both ε -cleavage sites, the mutations destabilize the T48 site more than L49 site. The increased accessibility of T48 for ε -cleavage can shift A β production towards A β 42, increasing the A β 42/A β 40 ratio. Such increased accessibility of T48 for ε -cleavage will lead to enhanced ε -cleavage at T48 relative to T49. This has been recently demonstrated by Dimitrov *et al.*, who reported a dramatic enhancement in the ratio of AICD49-99/AICD50-99 in V44A and other FAD mutants near the γ -secretase cleavage site³⁴. The proposed mechanistic hypothesis is also consistent with the report that FAD mutations within APP usually increase the A β 42/A β 40 ratio by modifying the product line preference of γ -secretase³⁵.

An alternate dimer structure of APPTM WT was reported in early 2012 by Nadezhdin *et al.*²⁸ This structure was also solved in DPC micelles and exhibited a similar dimer interface as in our structure, with the GXXXA as the dimerization motif. The Nadezhdin structure is significantly different from our structure,

Table 1 NMR and refinement statistics for APPTM structures.		
	WT	V44M
NMR distance and dihedral constraints (per monomer)		
Distance constraints	603	624
Total NOE	583	592
Intra-residue	410	404
Inter-residue	164	188
Sequential ($ i - j = 1$)	410	404
Medium range ($ i - j < 4$)	140	162
Long range ($ i - j > 5$)	24	26
Intermolecular	9	11
Hydrogen bonds	20	21
Total dihedral angle restraints	54	56
phi	27	28
psi	27	28
Dipolar coupling restraints	44	44
RDC from stretched gel	20	19
RDC from d(GpG)	24	25
Structure Statistics		
Violations (mean and s.d.)		
Distance constraints (Å)	0.035 $\pm$ 0.006	0.062 $\pm$ 0.008
Dihedral angle constraints (°)	0.664 $\pm$ 0.180	0.560 $\pm$ 0.247
Max. dihedral angle violation (°)	0.844	0.807
Max. distance constraint violation (Å)	0.041	0.070
Deviations from idealized geometry		
Bond lengths (Å)	0.003 $\pm$ 0.000	0.004 $\pm$ 0.000
Bond angles (°)	0.441 $\pm$ 0.024	0.623 $\pm$ 0.053
Impropers (°)	0.475 $\pm$ 0.043	0.535 $\pm$ 0.025
RDC		
RDC from stretched gel (Hz)	1.336 $\pm$ 0.182	1.504 $\pm$ 0.190
RDC from d(GpG) (Hz)	1.531 $\pm$ 0.329	2.702 $\pm$ 0.319
Average pairwise r.m.s.d. (20) (Å)		
Heavy	1.28 $\pm$ 0.34	1.31 $\pm$ 0.25
Backbone	0.79 $\pm$ 0.31	0.81 $\pm$ 0.26

as the RDC values used in our structure calculation are not consistent with the Nadezhdin structure (Supplementary Fig. S7). A major difference between our structure and the Nadezhdin structure is that we have a right-handed dimer, while they have a

Figure 3 | NMR structures and dimer interfaces of WT and V44M. The interface residues are colored green in WT structure (a) and magenta in V44M structure (b). Interfacial residues unique to WT is labelled in green letters while those unique to V44M is labelled in magenta. GXXXA is the dimerization motif in both structures, but the interface is shifted towards the C-terminus in V44M. (c) Overlay of the ribbon representation of structures of WT (red) and V44M (blue). V44M conserves the overall dimer fold, with an RMSD between the average structures of WT and V44M of 2.4 Å.

left-handed dimer. Their construct contained an additional 13 residues QKLFFAEDDVGs to the N-terminus of APPTM, which formed a nascent helix and could contribute to the structural discrepancy. Their dimer structures were built on 6 intermolecular NOEs between methyl groups from filtered experiments only. In contrast, our structures are based upon intermolecular NOEs derived from three differentially labelled NMR chimera samples, in addition to NOEs from filtered experiments (Fig. 2). We used a mixed sample of [U - ^{15}N ; U - ^2H] APPTM and unlabelled APPTM to obtain intermolecular NH-methyl NOEs. We also utilized a 1:1 mixture of [U - ^{15}N] labelled and [U - ^{13}C] labelled sample to yield unambiguous intermolecular NOEs between ^{15}N -H and ^{13}C -H. No RDC data were used in the calculation of the Nadezhdin structure while our structures were refined with RDCs determined in two alignment

Figure 4 | Average length of helical hydrogen bonds in APPTM WT and V44M. The average distances between the donor amide proton (residue $i+4$) and the acceptor carbonyl (residue i) in the TM helix in the 20 NMR structures and their standard deviations are plotted versus residue number of the donor amide. The error bars here are standard deviations of hydrogen bond lengths derived from 20 NMR structures. Overall, hydrogen bonds at the N-terminal half become shorter while hydrogen bonds at the C-terminal half become longer in V44M, suggesting that the V44M mutation tightens up the N-terminal half helix while loosening up the C-terminal half helix. In particular, the helical hydrogen bond involving T48 amide is significantly lengthened, consistent with its decreased amide chemical shift in V44M.

media, dGpG and stretched gel. RDC values can globally define NH orientations and avoid potential mistakes resulting from the local nature of NOE distance constraints.

A recent NMR structure of C99 has been published which includes APPTM within the sequence²³. C99 is known to dimerize through APPTM and is present in both monomer and dimer forms^{24,31,37}. Barrett *et al.* chose to solve the monomer structure of C99 in lyso-myristoylphosphatidylglycerol (LMPG) micelles and primarily focused on the cholesterol-binding properties of C99. Therefore, the C99 NMR structure represents an alternative functional state for APPTM from our APPTM dimer structure. Interestingly, Barrett *et al.* showed that one of the GXXXG motifs plays a key role in cholesterol binding, which is consistent with our observation that GXXXG motif is not involved in APPTM dimerization.

APPTM dimerization has been well-established, but the dimerization motif has been controversial. There are three potential dimerization motifs within APPTM: two GXXXG motifs and one GXXXA motif (Supplementary Fig. S1). Several groups provided evidence supporting GXXXG motifs as the key dimerization motif^{25,27,31,38,39}, while the current study, Nadezhdin *et al.*²⁸ and Gorman *et al.*²⁶ demonstrated that the GXXXA motif is the single most important motif for dimerization. The differences in the literature are likely due to variations in experimental conditions. Disparate membrane mimetics, for example, the different types of detergent, lipid and membrane system employed, can have dramatic impact on the dimerization mode. Indeed a solid-state NMR study has shown that lipid composition in multi-lamellar vesicle can significantly alter the conformation and dynamics of APPTM²⁹. Recently, Wang *et al.* published an MD simulation of APPTM dimerization in conjunction with TOXCAT dimerization assay and showed that APPTM may have multiple dimerization modes, in which both GXXXG and GXXXA can mediate dimerization³¹. Therefore, it is likely that APPTM may dimerize through various motifs under different physiological conditions and for different functions. The presence of cholesterol may induce a dimerization mode more amenable to γ -secretase cleavage, where GXXXA

Figure 5 | HD exchange shows that FAD mutants V44M and V44A destabilize the local conformations of T48 more than L49. (a) HD exchange rates for slowly exchanging residues in WT APPTM, V44M and V44A are plotted. Terminal residues (K28-M35 and L52-K55) exchange with D₂O completely within 1 h ($k_{ex} > 5$ per h) and their rates are not plotted. Exchange rates for T48 are significantly enhanced by V44A and V44M mutations while the increases for L49 are to a lesser extent. Increased k_{ex} values at A44 and T43 are likely due to the decrease in side-chain size from a valine to an alanine in V44A. The error bars are standard deviations that are derived from a Monte Carlo simulation assuming Gaussian distribution of random error in peak heights. **(b)** Novel mechanism of how FAD mutation V44M and V44A increase Aβ42/Aβ40 ratio. V44M and V44A open up T48 for the initial ε-cleavage by γ-secretase. The change in Aβ product line preference towards Aβ42 by FAD mutations increases Aβ42/Aβ40 ratio.

mediates dimerization and GXXXG motifs bind to cholesterol. As the γ-secretase cleavage progresses and the hydrophobicity of APPTM decreases, the dimerization motif may shift towards the N-terminus and be mediated by the GXXXG motifs, facilitating the exit of Aβ from the membrane.

In summary, we have shown that the V44M and V44A FAD mutations change the structure and dynamics of ε-cleavage sites. T48, the ε-cleavage site for Aβ42 generation, is likely more accessible to γ-secretase in the V44M and V44A mutants, leading to a shift towards the Aβ42 production line and to increased Aβ42/Aβ40 ratio. Such a structural mechanism may be applicable for other FAD mutants within APPTM and suggests that the ε-cleavage site may be a promising target for AD drug discovery.

Methods

Materials. Isotopically labelled compounds necessary for APPTM NMR sample production, such as ²H-labelled DPC, were purchased from Cambridge Isotope Laboratories.

Protein expression and purification. The APPTM gene was cloned into the pETM41 vector for expression as an MBP fusion protein and purified by an amylose affinity column followed by TEV protease digest³². The FAD mutants, V44M and V44A of APPTM was obtained through site-directed mutagenesis.

NMR spectroscopy. The NMR sample contains 0.2–0.5 mM of APPTM with 5% DPC, 25 mM sodium phosphate at pH 7.2 and 10% D₂O. NMR experiments for assignment and structure calculation were performed on either a 600 or 800 MHz

Bruker Advance II spectrometer equipped with cryogenic probes, at 313 K. Spectra were processed with nmrPipe software⁴⁰ and analysed using Sparky (T.D. Goddard and D.G. Kneller, SPARKY 3, University of California, San Francisco, CA). For APPTM WT and V44M, the backbone resonances were assigned using HNCACB and HNCOCACB. Side-chain assignments were carried out using ¹⁵N-TOCSY, (H)C(CCO)NH-TOCSY, H(CCCO)NH-TOCSY and HC(C)H-TOCSY experiments. Stereospecific assignment of side-chain valine and leucine was obtained by fractional 10% ¹³C labelling. Assignments of ε-methyls of methionines were accomplished by mutagenesis. Backbone assignment of V44A was obtained by an HNCA experiment.

NMR structure determination. CYANA3.0 (ref. 41) was used for initial structure calculation. Distance constraints were obtained from ¹³C and ¹⁵N-NOESY spectra. Intermolecular NOEs were specifically detected by both filtered experiments and appropriate experiments for chimera NMR samples (Fig. 2). The structure from CYANA with the lowest target function values were subject to refinement using XPLOR-NIH⁴² with RDC from stretched gel⁴³ and d(GpG)⁴⁴. The quality of the final structures was assessed with PSVS⁴⁵. 20 lowest energy conformers out of 200 refined structures were deposited into protein data bank (2LZ3 and 2LZ4). Ramachandran statistics for the 20 structures are 95.2% and 95.1% in the most favoured regions, 4.8% and 4.9% in the additionally allowed regions for WT and V44M, respectively, 0% in either generally allowed or disallowed regions.

HD exchange. APPTM were exchanged into D₂O using Zeba Spin Desalting Columns (Thermo Scientific). Samples were then monitored by ¹⁵N-¹H HSQC for up to 30 h at pH 7.2 and 298 K.

References

- Selkoe, D. J. Toward a comprehensive theory for Alzheimer's disease. Hypothesis: Alzheimer's disease is caused by the cerebral accumulation and cytotoxicity of amyloid beta-protein. *Ann. N. Y. Acad. Sci.* **924**, 17–25 (2000).
- Annaert, W. & De Strooper, B. A cell biological perspective on Alzheimer's disease. *Annu. Rev. Cell Dev. Biol.* **18**, 25–51 (2002).
- Hardy, J. & Selkoe, D. J. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**, 353–356 (2002).
- Burdick, D. *et al.* Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs. *J. Biol. Chem.* **267**, 546–554 (1992).
- Zhang, Y., McLaughlin, R., Goodyer, C. & LeBlanc, A. Selective cytotoxicity of intracellular amyloid beta peptide (1–42) through p53 and Bax in cultured primary human neurons. *J. Cell Biol.* **156**, 519–529 (2002).
- Mucke, L. *et al.* High-level neuronal expression of abeta 1–42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* **20**, 4050–4058 (2000).
- De Jonghe, C. *et al.* Pathogenic APP mutations near the gamma-secretase cleavage site differentially affect Abeta secretion and APP C-terminal fragment stability. *Hum. Mol. Genet.* **10**, 1665–1671 (2001).
- Suzuki, N. *et al.* An increased percentage of long amyloid-beta protein secreted by familial amyloid-beta protein-precursor (Beta-APP717) mutants. *Science* **264**, 1336–1340 (1994).
- Sinha, S. *et al.* Purification and cloning of amyloid precursor protein beta-secretase from human brain. *Nature* **402**, 537–540 (1999).
- Vassar, R. *et al.* Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286**, 735–741 (1999).
- Fortini, M. E. Gamma-secretase-mediated proteolysis in cell-surface-receptor signalling. *Nat. Rev. Mol. Cell Biol.* **3**, 673–684 (2002).
- Fraering, P. C. *et al.* Purification and characterization of the human gamma-secretase complex. *Biochemistry* **43**, 9774–9789 (2004).
- Haass, C. Take five—BACE and the gamma-secretase quartet conduct Alzheimer's amyloid beta-peptide generation. *EMBO J.* **23**, 483–488 (2004).
- Takami, M. *et al.* Gamma-secretase: successive tripeptide and tetrapeptide release from the transmembrane domain of beta-carboxyl terminal fragment. *J. Neurosci.* **29**, 13042–13052 (2009).
- Suzuki, N. *et al.* An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. *Science* **264**, 1336–1340 (1994).
- Maruyama, K. *et al.* Familial Alzheimer's disease-linked mutations at Val717 of amyloid precursor protein are specific for the increased secretion of A beta 42(43). *Biochem. Biophys. Res. Commun.* **227**, 730–735 (1996).
- Scheuner, D. *et al.* Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nat. Med.* **2**, 864–870 (1996).
- Lichtenthaler, S. F. *et al.* Mechanism of the cleavage specificity of Alzheimer's disease gamma-secretase identified by phenylalanine-scanning mutagenesis of the transmembrane domain of the amyloid precursor protein. *Proc. Natl Acad. Sci. USA* **96**, 3053–3058 (1999).

19. Kumar-Singh, S. *et al.* 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.* **9**, 2589–2598 (2000).
20. Hecimovic, S. *et al.* Mutations in APP have independent effects on Abeta and CTFgamma generation. *Neurobiol. Dis.* **17**, 205–218 (2004).
21. Ancolio, K. *et al.* Unusual phenotypic alteration of beta amyloid precursor protein (betaAPP) maturation by a new Val-715 --> Met betaAPP-770 mutation responsible for probable early-onset Alzheimer's disease. *Proc. Natl Acad. Sci. USA* **96**, 4119–4124 (1999).
22. Yin, Y. I. *et al.* Gamma-secretase substrate concentration modulates the A beta 42/A beta 40 ratio. *J. Biol. Chem.* **282**, 23639–23644 (2007).
23. Barrett, P. J. *et al.* The amyloid precursor protein has a flexible transmembrane domain and binds cholesterol. *Science* **336**, 1168–1171 (2012).
24. Beel, A. J. *et al.* Structural studies of the transmembrane C-terminal domain of the amyloid precursor protein (APP): does APP function as a cholesterol sensor? *Biochemistry* **47**, 9428–9446 (2008).
25. Kienlen-Campard, P. *et al.* 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.* **283**, 7733–7744 (2008).
26. Gorman, P. M. *et al.* Dimerization of the transmembrane domain of amyloid precursor proteins and familial Alzheimer's disease mutants. *BMC Neurosci.* **9**, 17 (2008).
27. Sato, T. *et al.* A helix-to-coil transition at the epsilon-cut site in the transmembrane dimer of the amyloid precursor protein is required for proteolysis. *Proc. Natl Acad. Sci. USA* **106**, 1421–1426 (2009).
28. Nadezhdin, K. D., Bocharova, O. V., Bocharov, E. V. & Arseniev, A. S. Dimeric structure of transmembrane domain of amyloid precursor protein in micellar environment. *FEBS Lett.* **586**, 1687–1692 (2012).
29. Lu, J. X., Yau, W. M. & Tycko, R. Evidence from solid-state NMR for nonhelical conformations in the transmembrane domain of the amyloid precursor protein. *Biophys. J.* **100**, 711–719 (2011).
30. Miyashita, N., Straub, J. E., Thirumalai, D. & Sugita, Y. Transmembrane structures of amyloid precursor protein dimer predicted by replica-exchange molecular dynamics simulations. *J. Am. Chem. Soc.* **131**, 3438–3439 (2009).
31. Wang, H. *et al.* Molecular determinants and thermodynamics of the amyloid precursor protein transmembrane domain implicated in Alzheimer's disease. *J. Mol. Biol.* **408**, 879–895 (2011).
32. Chen, W., Gamache, E., Richardson, D., Du, Z. M. & Wang, C. Y. Expression, purification, and reconstitution of the transmembrane domain of the human amyloid precursor protein for NMR studies. *Protein. Expr. Purif.* **81**, 11–17 (2012).
33. MacKenzie, K. R., Prestegard, J. H. & Engelman, D. M. A transmembrane helix dimer: structure and implications. *Science* **276**, 131–133 (1997).
34. Dimitrov, M. *et al.* Alzheimer's disease mutations in APP but not gamma-secretase modulators affect epsilon-cleavage-dependent AICD production. *Nat. Commun.* **4**, 2246 (2013).
35. Chavez-Gutierrez, L. *et al.* The mechanism of gamma-Secretase dysfunction in familial Alzheimer disease. *EMBO J.* **31**, 2261–2274 (2012).
36. Golde, T. E., Ran, Y. & Felsenstein, K. M. Shifting a complex debate on gamma-secretase cleavage and Alzheimer's disease. *EMBO J.* **31**, 2237–2239 (2012).
37. Botev, A. *et al.* The amyloid precursor protein C-terminal fragment C100 occurs in monomeric and dimeric stable conformations and binds gamma-secretase modulators. *Biochemistry* **50**, 828–835 (2011).
38. Munter, L. M. *et al.* Aberrant amyloid precursor protein (APP) processing in hereditary forms of Alzheimer disease caused by APP familial Alzheimer disease mutations can be rescued by mutations in the APP GxxxG motif. *J. Biol. Chem.* **285**, 21636–21643 (2010).
39. Munter, L. M. *et al.* GxxxG motifs within the amyloid precursor protein transmembrane sequence are critical for the etiology of Abeta42. *EMBO J.* **26**, 1702–1712 (2007).
40. Delaglio, F. *et al.* NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **6**, 277–293 (1995).
41. Guntert, P. Automated NMR structure calculation with CYANA. *Methods Mol. Biol.* **278**, 353–378 (2004).
42. Schwieters, C. D., Kuszewski, J. J., Tjandra, N. & Clore, G. M. The Xplor-NIH NMR molecular structure determination package. *J. Magn. Res.* **160**, 66–74 (2003).
43. Chou, J. J., Gaemers, S., Howder, B., Louis, J. M. & Bax, A. A simple apparatus for generating stretched polyacrylamide gels, yielding uniform alignment of proteins and detergent micelles. *J. Biomol. NMR* **21**, 377–382 (2001).
44. Lorieau, J., Yao, L. & Bax, A. Liquid crystalline phase of G-tetrad DNA for NMR study of detergent-solubilized proteins. *J. Am. Chem. Soc.* **130**, 7536–7537 (2008).
45. Bhattacharya, A., Tejero, R. & Montelione, G. T. Evaluating protein structures determined by structural genomics consortia. *Proteins* **66**, 778–795 (2007).

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Author contributions

C.W. and Y.-M.L. designed the research. C.W. supervised the project. E.G. made the overexpression plasmids and established the protein purification protocols. W.C. carried out most experiments and data analysis. D.J.R. assisted with NMR structure calculation. M.M.L. carried out analytical ultracentrifugation. J.X. assisted with HD exchange experiments. C.W. and W.C. wrote the paper.

Additional information

Accession codes: The NMR structural coordinates and constraints of the wild-type and V44M APP-TM proteins have been deposited in the protein databank under accession codes 2LZ3 (WT) and 2LZ4 (V44M).

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