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Pathogenic APP mutations near the γ-secretase cleavage site differentially affect Aβ secretion and APP C-terminal fragment stability

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Release of amyloid β (Aβ) from the amyloid precursor protein (APP) requires cleavages by β- and γ-secretases and plays a crucial role in Alzheimer's disease (AD) pathogenesis. Missense mutations in the APP gene causing familial AD are clustered around the β-, α- and particular γ-secretase cleavage sites. We systematically compare in primary neurons the effect on APP processing of a series of clinical APP mutations (two of which not characterized before) located in close proximity to the γ-secretase cleavage site. We confirm and extend previous observations showing that all these mutations (T714I, V715M, V715A, I716V, V717I and V717L) affect γ-secretase cleavage causing an increased relative ratio of Aβ42 to Aβ40. Taking advantage of these extended series of APP mutations we were able to demonstrate an inverse correlation between these ratios and the age at onset of the disease in the different families. In addition, a subset of mutations caused the accumulation of APP C-terminal fragments indicating that these mutations also influence the stability of APP C-terminal fragments. However, it is unlikely that these fragments contribute significantly to the disease process.

INTRODUCTION

Mutations in the amyloid precursor protein (APP) gene were the first to be recognized to cause early-onset autosomal-dominant Alzheimer's disease (AD). Since the initial report of a pathogenic APP mutation (1), 10 different APP mutations have been identified in a limited number of early-onset AD families (2–10; C.Van Broeckhoven, unpublished data). All mutations cluster near the proteolytic cleavage sites in APP and were shown to affect the release of amyloidβ (Aβ) (6–8,11–18; C.Van Broeckhoven, unpublished data). Release of Aβ involves successive cleavages by β-secretase (BACE) and γ-secretase. BACE cleaves APP between residues 671 and 672 (codon numbering of the APP770 isoform) or between residues 681 and 682 (19), leading to the secretion of the APP ectodomain (sAPPβ) in the extracellular medium and to the retention of a 12 kDa cell-bound C-terminal fragment (CTFβ or C99). Further cleavage of CTFβ by γ-secretase, predominantly occurring between residues 711 and 712 (γ40-cleavage), and to a lesser extent between residues 713 and 714 (γ42-cleavage), results in the secretion of, respectively, a 40 and 42 amino acid Aβ peptide (Aβ40 and Aβ42). The remaining 55–57 residue long CTF (CTFγ or C55/57, or AID:APP intracellular domain), is rapidly degraded (P.Cupers, W.Annaert and B.De Stooper, manuscript submitted for publication) and is only present at extremely low levels *in vitro* (20,21) and *in vivo* (22). The function of this fragment remains elusive. Alternatively, APP can be cleaved within the Aβ sequence by α-secretase, leading to the secretion of the APP ectodomain (sAPPα) and the retention of a 10 kDa CTF (CTFα or C83). Subsequent cleavage of CTFα by γ-secretase leads to the production of a 3 kDa N-truncated amyloid peptide, named p3.

Presenilin (PS) proteins are required in γ-secretase-mediated cleavage of APP, since deletion of the PS1 gene inhibits Aβ generation (23). Moreover, the finding that mutagenesis of critical aspartate residues (D257 and D385) in PS1 causes reduced Aβ secretion and accumulation of APPCTFs led to the hypothesis that PS1 may exert an aspartyl protease activity and that it might be identical to γ-secretase (24). In addition to most APP mutations, PS interferes with γ-secretase activity in such a way that production of the highly amyloidogenic Aβ42 is increased (25). Consequently, a leading hypothesis for AD etiology is 'the amyloid cascade hypothesis' (26) stating that increased Aβ42 secretion represents a gain of misfunction.

However, it was reported recently that the APP V715M mutation affects Aβ40 rather than Aβ42 secretion (7). Moreover, the artifical putative active site D257A PS1 mutant causes accumulation of APPCTFs without a concomitant reduction in Aβ secretion, indicating that accumulation of

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APPCTFs is not necessarily a direct consequence of decreased Aβ production (27).

Recently, several novel mutations, clustered at the γ-secretase processing site of APP, have been identified (6–10). To compare their relative effects on γ-secretase cleavage, we expressed human APP695 with or without the clinical mutants T714I, V715M, V715A, I716V, V717I and V717L in primary cultures of cortical neurones and analysed the full scope of APP metabolites. We demonstrate that these mutations lead to significantly increased relative Aβ42/Aβ40 concentrations and that the level of increase is inversely correlated with the age at onset of AD. Furthermore, some but not all C-terminal APP mutations also influenced the turnover of APPCTFs, resulting in the accumulation of APPCTFs.

RESULTS

Effect on Aβ secretion

We systematically analysed the effect of a series of clinical mutations near the γ-secretase cleavage site of APP. To this end, we expressed human wild-type APP or APP containing either the Austrian T714I (8), French V715M (7), German V715A (C.Van Broeckhoven, unpublished data), Florida I716V (6), Indiana V717L (9) or London V717I (1) mutation in primary cultures of neurons. We specifically immunoprecipitated Aβ40 or Aβ42 from the conditioned medium using antibody FCA3340 or antibody FCA3542, respectively (28). All APP C-terminal mutations with the exception of V715M, increased Aβ1–42 secretion (Fig. 1). The increase in Aβ1–42 ranged from 1.54-fold for T714I to 2.71-fold for V717L (Fig. 1C). This was accompanied by an increase in the N-truncated Aβ-isoforms ending at residue 42 (Aβx-42). These isoforms run in the gels as doublet bands (Fig. 1B), and are presumably generated by alternative β-secretase activity at amino acid residue 11 (19,29) or by α-secretase activity at amino acid residue 17 (30) of the Aβ sequence. Aβ1–40, on the other hand, is decreased with most mutations. The T714I, V715M and V715A mutations reduce Aβ1–40 secretion most drastically to 20, 30 and 55% of the wild-type levels, respectively. The V717I and V717L mutations affect Aβ40 secretion to a lesser extent and the I716V mutation has apparently no effect on Aβ40 secretion (Fig. 1C). The secretion of N-truncated Aβ40 isoforms (Aβx-40) was decreased to an extent comparable to Aβ1–40.

Hence, when the Aβ1–42/Aβ1–40 ratio is compared to wild-type APP (arbitrarily set equal to 1) for each mutation, this ratio is increased in all APP mutations. This increase ranges from 1.89-fold for V717I to 8.20-fold for T714I (Fig. 2A). Moreover, the Aβ1–42/Aβ1–40 ratio correlates inversely (r = –0.84) with the mean age of onset for the different mutations (http://molgen-www.uia.ac.be/ADMutations/) (Fig. 2B).

Effect on secretion of sAPP

We immunoprecipitated the secreted APP ectodomain from the conditioned medium of neurons transduced with SFV expressing wild-type or mutant APP using antibody 207. No significant differences in levels of sAPP between any of the mutants and wild-type APP were observed (data not shown), in agreement with the assumption that those mutations mainly affect γ-secretase processing of APP.

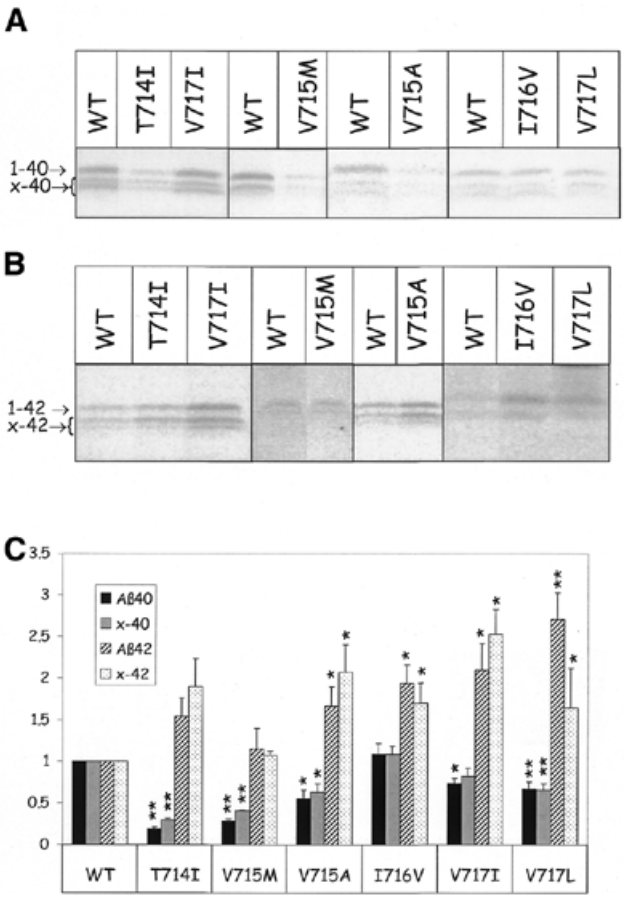

Figure 1. Effect of APP C-terminal mutations on Aβ secretion. Primary neuronal cultures were transduced with recombinant SFV expressing the indicated APP mutants. Cells were metabolically labelled with [^{35}S]methionine for 4 h and culture media were collected. Immunoprecipitation with antibodies FCA3340 (1:200), specifically recognizing Aβ40 (**A**), or FCA3542 (1:200), specifically recognizing Aβ42 (**B**), was performed on the culture media. Precipitates were separated by 4–12% SDS–NuPAGE and detection of radioactive bands was performed using PhosphorImaging (**C**). Values are expressed relative to wild-type, which is arbitrarily set equal to 1, and represent the means ± SEM of three experiments. *$P < 0.05$; **$P < 0.0001$.

Effect on the generation of APPCTFs

The CTFs (CTFα and CTFβ) that remain associated with the cells after α-, or β-secretase cleavage of APP were immunoprecipitated using antibody B11/4, directed against the last 20 amino acids of the cytoplasmic domain of APP. Mutations at codons 714 (T714I) and 717 (V717I and V717L) caused increased levels of APPCTFs (1.41–2.17-fold). The other mutations, at codons 715 (V715A and V715M) and 716 (I716V), displayed similar or even slightly decreased levels of APPCTFs compared with wild-type APP (Fig. 3).

We further investigated whether the accumulation of APPCTFs observed with the T714I mutation was the consequence of reduced degradation or increased production. Therefore pulse-chase experiments were performed and the turnover rate of wild-type and mutant CTFs was assessed. As can be

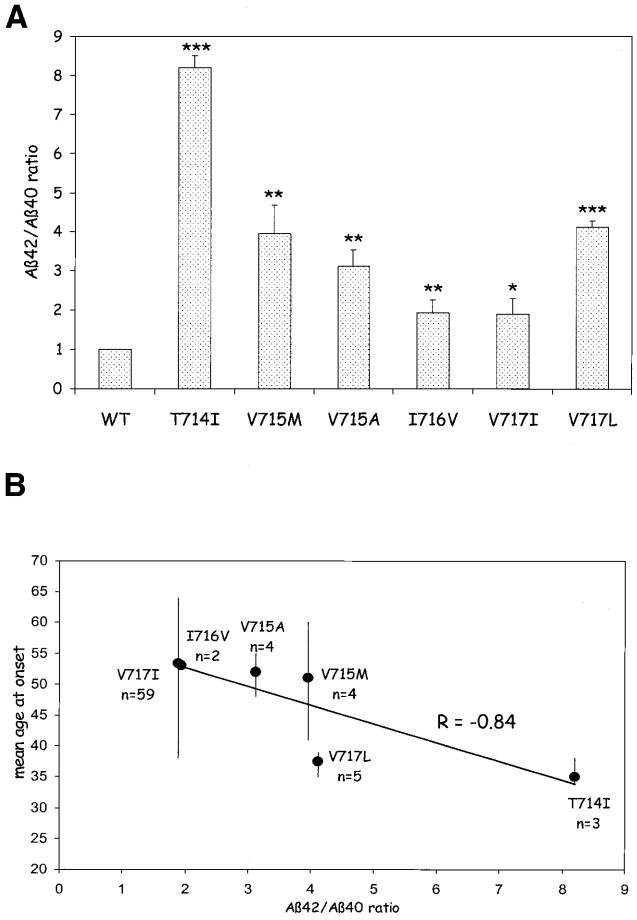

Figure 2. Effect of APP C-terminal mutations on Aβ42/Aβ40 ratio. Primary neuronal cultures were transduced with recombinant SFV expressing the indicated APP mutants. Secretion of Aβ1–42 and Aβ1–40 was measured as described in Figure 1. (**A**) Mean Aβ1–42/Aβ1–40 ratio, calculated for three independent experiments, compared to wild-type, which is arbitrarily set equal to 1. *$P < 0.1$; **$P < 0.05$; ***$P < 0.0001$. (**B**) Inversely linear relationship between the Aβ42/Aβ40 ratio and the mean age at onset of AD. Mean age of onset was calculated for each mutation including data from all available families (http://molgen-www.uia.ac.be/ADMutations/). Also indicated are age of onset range and number of patients (*n*).

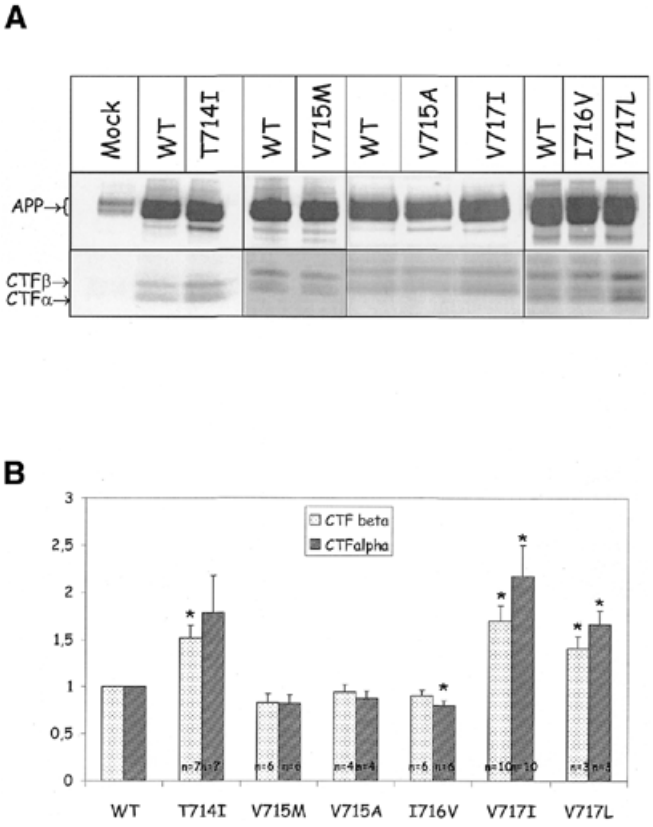

Figure 3. Effect of C-terminal APP mutations on production of APPCTFs. (**A**) Primary neuronal cultures were transduced with recombinant SFV expressing the indicated APP mutations. Cells were metabolically labelled with [^{35}S]methionine for 4 h, cells were washed and lysed. Immunoprecipitation with antibody B11/4 (1:200) against the APP cytoplasmic tail was performed on the cell lysates. Precipitates were separated by 4–12% SDS–NuPAGE and detection of radioactive material was performed using PhosphorImaging (**B**). Data are expressed relative to wild-type and represent means ± SEM of the indicated number (*n*) of experiments. *$P < 0.01$.

appreciated from Figure 4A and B, the turnover rate of the APP holoprotein is more or less identical for wild-type and mutant APP. In contrast, the turnover rates of wild-type APPCTFS or APPI716VCTFs is faster than the turnover rates of the APPCTFs containing the T714I or V717I mutation (Fig. 4B). Three hours after the pulse labeling, the level of APPCTFs is clearly decreasing with wild-type APP while APPCTFs continue to accumulate with the T714I or the V717I mutation. The degradation rate of I716V mutant APPCTFs was identical to the wild-type.

DISCUSSION

The common feature of all APP C-terminal mutations tested in the current study, is the significant increase in the relative ratio of Aβ42 to Aβ40. By far the largest increase (8.20-fold) was observed with the T714I mutant, corroborating our previous data in HEK-293 cells (8). It is striking that this mutant is also clinically the most aggressive one and displays an unusually early age of onset of 34 years, compared to other APP mutations. It will be interesting to test this mutant in APP transgenic mice, anticipating the rapid development of amyloid plaques and disease in these animals. More importantly, by systematically analysing an extended series of C-terminal APP mutants in a single neuronal expression paradigm, we were able to demonstrate for the first time a clear inverse correlation between Aβ42/Aβ40 ratio and age of onset for the APP mutations, which strongly supports (a variant of) the 'amyloid peptide hypothesis'. It should be mentioned that in APP V717I families age of onset is apparently modulated by the APOE genotype with APOE4 decreasing and APOE2 increasing the age of onset (31,32). The other APP mutations were found in single families and APOE data is too sparse to allow analysis of APOE effect on age of onset (http://molgen-www.uia.ac.be/ADMutations/). Our correlation data also contrast with the observation that in PS1 families, age of onset is not inversely correlated with Aβ42 secretion (33–35). In these families, age of onset is also not influenced by APOE genotype (36). Remarkably, the observed correlation only holds true when the relative levels of Aβ42 to Aβ40 are considered. It is not

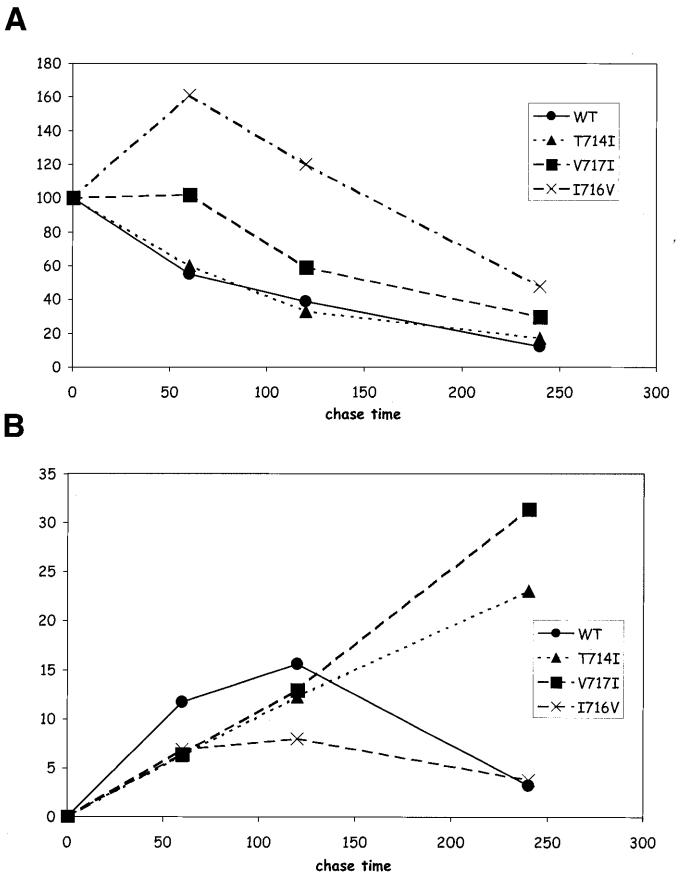

Figure 4. Pulse–chase analysis of wild-type and mutant full-length APP. Primary neuronal cultures were transduced with recombinant SFV expressing the indicated APP mutations. Cells were pulse-labelled with [^{35}S]methionine for 30 min, followed by a chase period of 0–240 min. Cell extracts were immunoprecipitated using antibody B11/4 (1:200) and separated using 4–12% SDS–NuPAGE. Detection of radioactive material was performed using PhosphorImaging. Quantification of the radioactive bands is shown in (**A**) for the APP holoprotein and in (**B**) for APPCTFs. The radioactive signals are normalized to the signal obtained for APP holoprotein at time zero. Data are representative for two independent experiments.

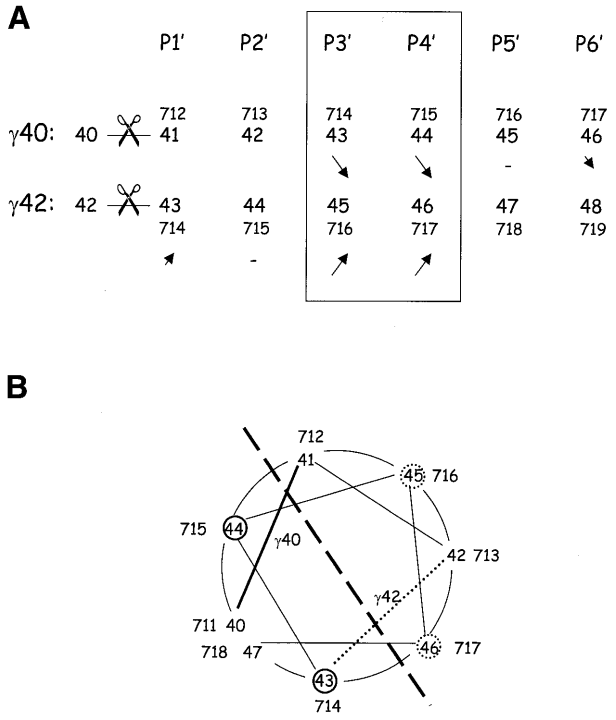

Figure 5. α-Helicoidal model for APP. (**A**) The positions of the mutated residues relative to the cleavage sites for γ-secretase are displayed. Amino acid numbering is indicated for APP holoprotein (712–718) and for Aβ (41–48). Arrows below the residue number indicate the effect of the mutation on secretion of the corresponding Aβ isoform. (**B**) Top view of a spatial model for the putative α-helical conformation [adapted from Lichtenthaler *et al.* (38)] of part of the transmembrane region of APP near the γ-secretase cleavage site is shown. Residues that, when mutated, mainly affect the γ40 cleavage (bold line) are fully encircled. Residues that when mutated affect predominantly γ42 cleavage (dashed line) are encircled with dashes. Note that both types of residues are located at opposite sides of the helix (separated by the bold dashed line).

completely clear why absolute amounts of Aβ42 do not correlate with age at onset of disease. A 'protective' role of Aβ40 seems unlikely since Aβ40 levels by themselves are not correlated with age at onset of disease (data not shown).

At the level of γ-secretase processing, the different mutations apparently have slightly different effects. Mutations at codons 714 (T714I) and 715 (V715A and V715M) appear to primarily affect the γ40-cleavage and cause a decreased secretion of Aβ40. Mutations at codons 716 (I716V) and 717 (V717I and V717L) predominantly affect the γ42-cleavage and increase Aβ42 secretion. Hence, pathogenic APP mutations exert their largest effect on the cleavage site which is located two or three residues more N-terminal to the substitution (Fig. 5A). This suggests that the residues in the P3′ and P4′ positions are of particular importance for the exposure of the scissile bounds to the catalytic sites of the γ-secretase protease(s). Remarkably, the drastic decreases in Aβ40 secretion observed with the mutations at codons 714 and 715 are not clearly accompanied by a large increase in Aβ42 secretion. However, as we demonstrated previously for the APP T714I mutation (8), Aβ isoforms ending at residues 39, 38 and to a lesser extent 37 may counterbalance the decrease in Aβ40. Overall, it follows that the ratio of Aβ42 to Aβ40 is the best indicator of the severity of the disease in patients with APP mutations.

Unexpectedly, APP C-terminal mutations also influence the levels of APPCTFs. The T714I, V717I and V717L mutations cause the accumulation of APPCTFs in neurons, whereas the V715A, V715M or I716V mutations have no significant effect on APPCTF levels. Both α- and β-secretase-generated APPCTFs increase approximately to the same extent. This elevation is not clearly accompanied by increased secretion of the sAPP, indicating that the accumulation of APPCTFs is not the direct result of increased α- or β-secretase processing of APP. Increased levels of APPCTFs could also result from reduced γ-secretase activity (23). It could be envisaged that the presence of the C-terminal APP mutation reduces γ-secretase activity. Since Aβ40 is the major Aβ isoform produced, a specific reduction of the γ40 cleavage could theoretically explain the accumulation of APPCTFs while maintaining Aβ42 levels at a pathogenic level. However, the APP mutations that drastically decreased Aβ40 secretion (V715A and V715M) did not display APPCTF accumulation, whereas the others (V717I and V717L) did not significantly affect Aβ40

secretion but accumulated APPCTFs. Therefore, the effect of the latter mutations on APPCTF metabolism must occur independently from the γ-secretase cleavage step. Our pulse–chase data indicated a reduced turnover rate of these mutant CTFs, suggesting that these APP mutations not only affect γ-secretase processing, but also the catabolic pathways that degrade these fragments. Accumulation of APPCTFs was shown before with the Swedish and London APP mutants K670N/M671L, V717I, V717F and V717G (37), in agreement with our findings. McPhie *et al.* (37) proposed that APPCTFβ is linked to a signalling transduction pathway. Accumulation of APPCTFβ was speculated to disrupt this pathway, leading to cytoskeletal abnormalities and neurodegeneration. However, our data show that accumulation of APPCTFs is not a consistent feature of all APP mutants causing AD. In fact, mutations V717I and I716V result in a very similar mean age of onset, whereas APPCTFs accumulate with the former but not with the latter. Therefore, accumulation of APPCTFs is likely a less important factor in the overall pathogenesis of the disease. It would be of interest to investigate these different mutations at the pathological level in the brain of patients. Unfortunately, no material is yet available from patients with mutations that do not cause APPCTF accumulation, i.e. mutations at positions 715 or 716 of the APP sequence.

Finally, we propose a molecular model that integrates our observations. If one presumes an α-helical conformation for the transmembraneous part of APP (38) (Fig. 5B), then the mutations at residues 714 and 715, which primarily affect γ40-secretase cleavage, are located at one side of the helix, whereas mutations at residues 716 and 717, which predominantly affect γ42-secretase cleavage, are located at the opposite side. It should be noted that the mutations at residues 714 and 717, leading to accumulation of APPCTFs, are located at one side of the helix, whereas mutations at residues 715 and 716, which do not lead to accumulation of APPCTFs are at the other side (Fig. 5B). Our recent unpublished data (W.Annaert and B.De Strooper) demonstrated that the region in APP spanning the currently investigated clinical mutations is involved in the direct binding of APP to PS1. We speculate, therefore, that the investigated clinical mutations cause subtle changes in the binding interaction between PS1 and APP, and therefore mainly affect exposure of the scissile bonds to the catalytic site of γ-secretase The abnormal accumulation of APPCTFs observed with some of the APP mutants could also fit this hypothesis, since the PS1 D257A catalytic site mutant leads to accumulation of APPCTFs without the concomitant reduction in Aβ production (27).

MATERIALS AND METHODS

Preparation of recombinant Semliki Forest virus

The Austrian T714I (8), German V715A (C.Van Broeckhoven, unpublished data), French V715M (7), Florida I716V (6) and Indiana V717L (9) mutations were introduced in human wild-type APP cDNA (695 isoform) by site-directed mutagenesis using appropriate oligonucleotides and the Quick-change mutagenesis system (Stratagene, La Jolla, CA). The mutant APP cDNA was subsequently cloned into the *Sma*I-site of pSFV-1 (Gibco BRL, Bethesda, MD). Recombinant Semliki Forest viruses were produced as described (39).

Primary neuronal cultures

Primary cortical neurons were isolated from E14 embryonic mice as described (39). Briefly, after dissection of the brain and dissociation of the cells by trypsinization, cells were plated on poly-lysine coated dishes and incubated in neurobasal medium + B27 supplement. Proliferation of non-neuronal cells was prevented by adding 5 μM cytosine arabinoside.

Transduction of primary neurons

Recombinant SFV was diluted 1:10 in conditioned culture medium and added to 3–5-day-old primary cortical neurons. After 1 h, medium was replaced with normal culture medium and transduction continued for 2 h. Medium was then replaced with methionine-free medium containing 100 μCi [^{35}S]methionine (ICN, Irvine, CA). After 4 h of metabolic labelling, culture supernatants were collected, cells were washed in PBS and finally lysed in DIP buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), containing protease inhibitors (5 mM EDTA, 100 U/ml trasylol, 1 μg/ml pepstatin).

Pulse–chase

Neurons were transduced with recombinant SFV as described above. Neurons were pulse-labeled with 100 μCi [^{35}S]methionine for 30 min and chased for 60, 120 and 240 min. Cell extracts were processed as described below.

Immunoprecipitation and SDS–PAGE

Antibodies directed against Aβ, 4G8 (1:200, Senetek, Napa, CA; recognizes total Aβ), FCA3340 (1:200, specifically recognizes Aβ40) (28) or FCA3542 (1:200, specifically recognizes Aβ42) (28) or to the secreted ectodomain of APP (antibody 207, 1:200) (40) were added to the culture supernatants and immune complexes were recovered with protein G–Sepharose. Antibody B11/4 was used to precipitate α and β C-terminal fragments of APP from the cell extracts (41). Immunoprecipitated material was resolved on a 4–12% NuPAGE gel (Novex; Invitrogen, Carlsbad, CA) run with MES buffer. The intensity of the radioactive bands was quantified using a phosphoimager (Molecular Dynamics, Sunnyvale, CA) and the ImageQuant software package. All quantitative data were normalized to the signal obtained for the corresponding APP holoprotein to compensate for variations in expression between culture dishes or between the different APP mutants used.

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