

Amyloid precursor protein mutation E682K at the alternative β -secretase cleavage β' -site increases A β generation[†]

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BACE1 cleaves the amyloid precursor protein (APP) at the β -cleavage site (Met₆₇₁–Asp₆₇₂) to initiate the generation of amyloid peptide A β . BACE1 is also known to cleave APP at a much less well-characterized β' -cleavage site (Tyr₆₈₁–Glu₆₈₂). We describe here the identification of a novel APP mutation E682K located at this β' -site in an early onset Alzheimer's disease (AD) case. Functional analysis revealed that this E682K mutation blocked the β' -site and shifted cleavage of APP to the β -site, causing increased A β production. This work demonstrates the functional importance of APP processing at the β' -site and shows how disruption of the balance between β - and β' -site cleavage may enhance the amyloidogenic processing and consequentially risk for AD. Increasing exon- and exome-based sequencing efforts will identify many more putative pathogenic mutations without conclusive segregation-based evidence in a single family. Our study shows how functional analysis of such mutations allows to determine the potential pathogenic nature of these mutations. We propose to classify the E682K mutation as probable pathogenic awaiting further independent confirmation of its association with AD in other patients.

INTRODUCTION

Mutations in the amyloid precursor protein (APP) gene cause early onset autosomal-dominant Alzheimer's disease (AD) (Brouwers et al, 2008; De Strooper & Annaert, 2010; Selkoe, 2001; Wilquet & De Strooper, 2004). Pathogenic mutations (<http://www.molgen.ua.ac.be/ADMutations/>) located close to

major APP cleavage sites (β - and γ/ϵ -sites) can increase total A β production, but most affect the ratio of different A β peptides without increasing total A β , favouring a relative increase in A β_{42} versus other species (Bentahir et al, 2006; Citron et al, 1992; De Jonghe et al, 2001; Di Fede et al, 2009; Kwok et al, 2000; Scheuner et al, 1996; Suzuki et al, 1994). A β_{42} and A β_{40} are the main components of the senile plaques in AD brain

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[†]This work is dedicated to the late Mrs Anna Vanluffelen.

parenchyma and the amyloid angiopathy in the cerebral blood vessels. Some other mutations have been identified in the Aβ sequence itself. Those affect either the generation of Aβ by the secretases, the aggregation properties of the Aβ peptide or its proteolytic degradation (Betts et al, 2008; Nilsberth et al, 2001; Ono et al, in press; Tomiyama et al, 2008; Tsubuki et al, 2003).

BACE1, a membrane-bound aspartic protease, is the β-secretase, which cleaves APP at the β-site Met₆₇₁-Asp₆₇₂ of APP (Asp₁ of the Aβ sequence; Hussain et al, 1999; Lin et al, 2000; Sinha et al, 1999; Vassar et al, 1999; Yan et al, 1999). This

cleavage generates the APP carboxyterminal fragment C99, which is a substrate for the γ-secretase complex, an intramembrane cleaving protease (De Strooper, 2003). γ-Secretase processing of C99 yields a mixture of Aβ peptides including Aβ₁₋₃₈, Aβ₁₋₄₀, and Aβ₁₋₄₂ as the most abundantly detected species in cell culture and biological fluids (Takami et al, 2009).

BACE1 cleaves APP in addition at a β'-site, a secondary cleavage site between Tyr₆₈₁ and Glu₆₈₂ (Glu₁₁ of Aβ) to generate C89, which is further processed by γ-secretase to produce truncated Aβ_{11-40/42} species. BACE1 cleavage at the

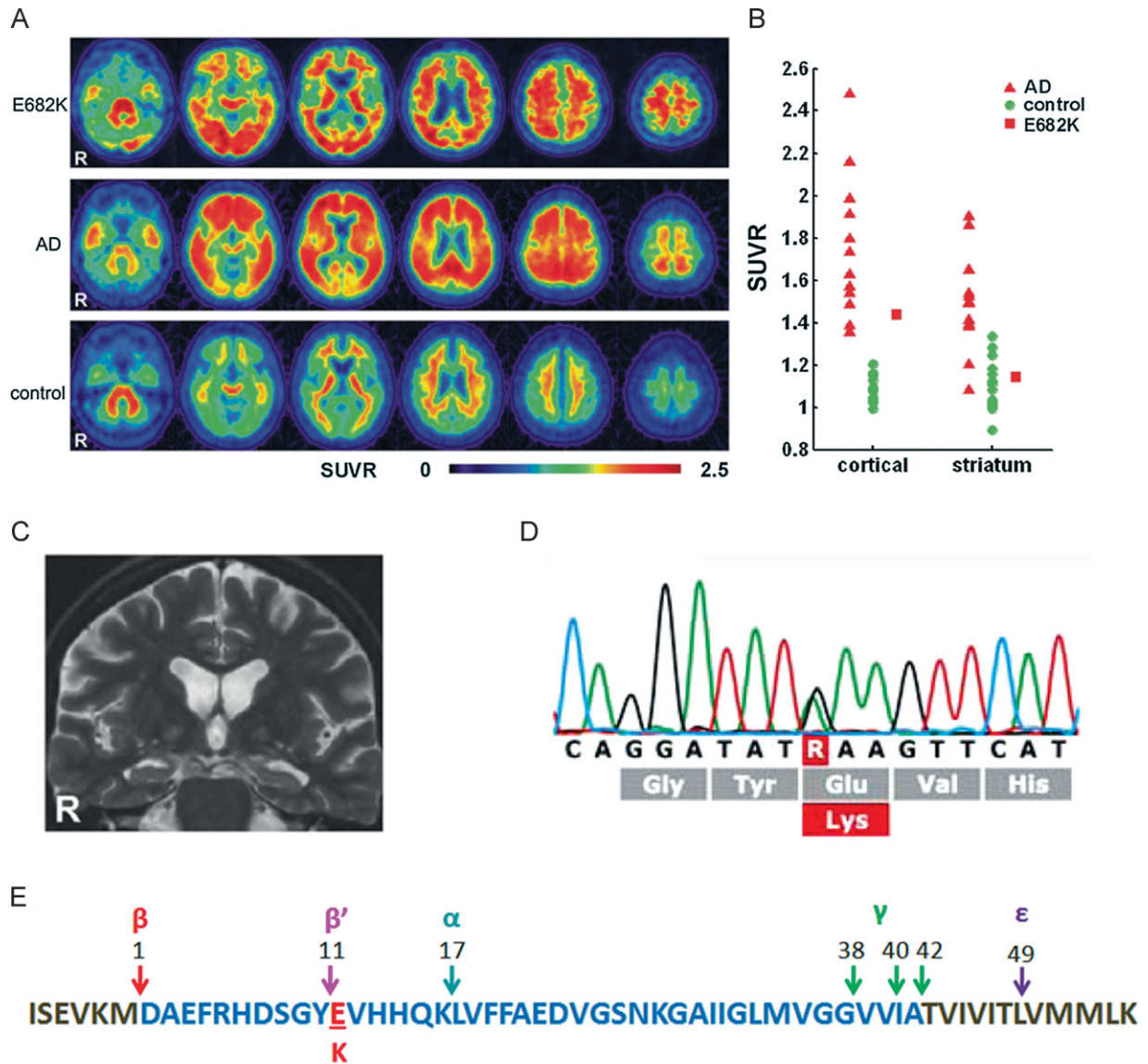


Figure 1. Clinical and genetic studies of a single case of an AD patient carrying the APP E682K mutation.

- A. Amyloid imaging using [¹¹C] Pittsburgh compound-B (PIB). The PIB SUVR (standardized uptake value ratio) image of index patient E682K compared to a mean image of 13 AD patients with increased cortical PIB uptake and 13 elderly controls with normal aspecific uptake, all set to the same scale.
- B. Plot of mean SUVR values in the composite cortical and striatal VOI.
- C. Magnetic resonance imaging (MRI) of the index patient showing hippocampal atrophy.
- D. DNA sequencing revealed the heterozygous APP E682K mutation.
- E. Schematic representation of APP E682K mutation and secretase cleavage sites, numbers were indicated according to Aβ sequence.

β' -site was originally discovered in cell cultures overexpressing this protease (Vassar et al, 1999). An *in vitro* study showed that purified BACE1 cleaves synthetic peptides mimicking the sequence around the human APP β' -site in an enzymatic assay, but the enzymatic efficiency was lower than towards peptides containing the 'canonic' β -site sequence (Yang et al, 2004). Additional evidence suggested that the relative abundance of BACE1 cleavage at these two adjacent sites is governed by the expression levels of the protease: when BACE1 levels are low, β -site cleavage products are the major species, when BACE1 levels are high, β' -site cleavage products become predominant (Creemers et al, 2001; Qahwash et al, 2004). These findings were also taken to suggest that β' -site processing was only a minor event in APP processing (Creemers et al, 2001). The abundance of the β' -site cleavage for human APP processing as well as the functional significance of this alternative β -secretase cleavage site remains, therefore, elusive.

In this work, we identify a novel and unusual APP mutation in a Belgian patient showing early onset AD and seen in the University Hospital in Leuven. This mutation -E682K- is located at the β' -site within the A β sequence (Fig 1F). We examined the effect of the E682K mutation on the proteolytic processing of APP and found that this mutation caused significant increases in total A β and in A $\beta_{1-42/40}$ levels. We further analysed APP processing in neuronal cultures by short metabolic labelling experiments demonstrating that β' -site cleavage is a major processing event of wild-type (WT) human APP in neuronal cultures. The E682K mutation blocked this processing step and consequentially shifted BACE1 cleavage towards the β -site. The data demonstrate the functional significance of β' -site cleavage in preventing overproduction of A β , which may potentially cause AD.

RESULTS

Clinical description of the index patient carrying the APP E682K mutation

The index patient presented at the Memory Clinic, University Hospitals Leuven, at the age of 50 years with a prior diagnosis of early onset clinically probable AD. Around the age of 47, she had developed symptoms of depression followed by gradually progressive cognitive decline with significant impact on her instrumental activities of daily living. At presentation, the index patient was on citalopram 10 mg twice per day and donepezil 10 mg once per day. Neuropsychological evaluation of the patient revealed a significant episodic memory deficit (Auditory Verbal Learning Test total learning 22/75, delayed recall 0/15; Logical Memory Wechsler Memory Scale: 4/23 literal elements, 2/5 meaningful elements), working memory deficit (Benton Visual Retention test 8/15) and executive dysfunction on the Stroop and Trail Making Test, together with word finding problems (Boston Naming test score 38/60). Magnetic resonance imaging T2-weighted sequence revealed bilateral hippocampal volume loss (Fig 1C). The cortical pattern of 11C-PIB uptake (Fig 1A and B) was closely similar to what is typically seen in clinically probable AD (Klunk et al, 2004; Nelissen et al, 2007). In contrast to what has been previously reported in

Table 1. A β and tau protein levels in cerebrospinal fluid

	Control	AD control	Patient (E682K)
n (Male/female)	7(5/2)	10 (6/4)	
Age	62.3 \pm 4.4	66.6 \pm 5.0	51
A β_{1-42} (pg/ml)	986.8 \pm 255.8	368.2 \pm 63.7	165
Total-tau (pg/ml)	168.7 \pm 63.7	925.8 \pm 487.8	834
phospho-tau (pg/ml)	57.8 \pm 16.17	122.2 \pm 34.1	104

Values are mean \pm SD.

presenilin 1 mutation carriers (Klunk et al, 2007), striatal uptake was within the normal range, similarly to what we observed for the K724N APP mutation (Theuns et al, 2006). Analysis of cerebrospinal fluid revealed decreased A β_{42} and increased total and 181T-phosphorylated tau (Table 1), a pattern corresponding to AD. Sequencing of the APP gene revealed a G to A substitution at g.278228G, which is predicted to result in an amino acid substitution at codon 682 (E682K). Such mutation was absent in 940 control samples. Her Apolipoprotein E genotype was ϵ 3/ ϵ 3.

The patient is from a small family. Her father received a diagnosis of dementia at the age of 75 and died at the age of 83 years. His genetic status is unknown as no sample was available for genomic analysis. The patient's brother, however, is clinically, at the age of 53, an asymptomatic carrier. We considered the possibility that the APPE682K mutation could have a variable penetrance in this family. A strong variation in age of onset (range 45–88 years) has previously been observed with AD causing mutations in *PSEN2* as well (Sherrington et al, 1996). We thus turned to functional assays to evaluate whether the identified mutation could be a genetic risk factor for AD or not.

E682K mutation increased A β generation

We introduced the E682K mutation by site directed mutagenesis into human WT APP695 and expressed WT or mutant APP in mouse primary neuronal cultures and in transiently transfected CHO cells. The expression levels of WT and mutant full length APP were checked by Western blotting, and neuronal or CHO cell cultures expressing similar amounts of APP were subjected to further analysis. Conditioned media were analysed using A β enzyme-linked immunosorbent assay (ELISAs; Table 2). In both

Table 2. A β_{1-42} and A β_{1-40} levels as well as A β_{1-42} /A β_{1-40} ratio in conditioned media from transfected CHO cells or transduced neurons

	A β_{1-42} (pg/ml)	A β_{1-40} (pg/ml)	A β_{1-42} /A β_{1-40} ratio
CHO cells			
APPWT	37.7 \pm 1.2	355.6 \pm 17.2	0.1065 \pm 0.0051
E682K	94.6 \pm 4.7	604.6 \pm 18.0	0.1563 \pm 0.0049**
A692G (Flemish)	77.5 \pm 2.4	621.3 \pm 27.2	0.1251 \pm 0.0039*
Primary neurons			
APPWT	60.0 \pm 1.9	546.3 \pm 14.3	0.1101 \pm 0.0046
E682K	189.9 \pm 5.1	1005.3 \pm 10.1	0.1890 \pm 0.0040**
A692G (Flemish)	131.7 \pm 1.5	1049.2 \pm 42.7	0.1257 \pm 0.0037*

Data were normalized to APP expression level (** $p < 0.01$, * $p < 0.05$; mean \pm S.E.M.; $n = 3$).

neuronal and CHO cell cultures, the E682K mutation caused a two- to three-fold increase in A β_{1-40} and A β_{1-42} levels, but also a slightly higher A β_{1-42} /A β_{1-40} ratio (Table 2). The alterations are quantitatively and qualitatively comparable to those caused by the previously characterized disease-causing 'Flemish' A692G mutation, which also caused increases in the A β levels and a slightly higher A β_{1-42} /A β_{1-40} ratio (Table 2; De Strooper et al, 1995; Haass et al, 1994; Hendriks et al, 1992; Nilsberth et al, 2001; Suzuki et al, 1994; Tian et al, 2010).

E682K mutation enhanced β -site cleavage of APP

We next analysed the effects of the E682K mutation on APP processing in further detail. In primary neuronal cultures, this mutation increased C99 and sAPP β levels two- to three-fold (Fig 2), which correlates well with the overall increases in A β levels as measured by ELISAs. Similar effects were observed in transiently transfected CHO cells, in which the E682K mutation caused a two- to three-fold increase in C99 and sAPP β levels (Fig S1 of Supporting Information). These data show that the E682K mutation increased A β generation by favouring the β -site cleavage of APP. In contrast to the E682K mutation, the 'Flemish' A692G mutation did not significantly affect the β -secretase processing (as measured by C99 and sAPP β generation), confirming that the increased A β generation with this mutant is caused by a different mechanism. It has indeed been shown that the 'Flemish' mutation affects an inhibitory domain in the APP sequence that modulates γ -secretase activity (Tian et al, 2010).

E682K mutation blocked the β' -site cleavage, which is a major processing event of human APP in neuronal cultures

We further examined how the E682K mutation might enhance β -site cleavage. This mutation is located at the previously identified β' -site in APP and the WT residue Glu₁₁ is known to occupy the P1' subsite of BACE1 involved in β' -site cleavage. A previous kinetic study has shown that the P1' subsite of BACE1 favours several residues including Asp, Glu, Met, Ala, Ser, and

Gln, but that positively charged residues like Lys or Arg decrease affinity (Turner et al, 2001). The E682K mutation was thus predicted to block the β' -site cleavage. However, until now, β' -cleavage of human APP is considered as a minor processing event in cells expressing endogenous levels of BACE1, and has been mainly documented in cells overexpressing BACE1 (Creemers et al, 2001; Huse et al, 2002; Liu et al, 2002; Qahwash et al, 2004).

Here, we reanalyse the metabolism of human APP in neuronal cultures. We expressed WT or mutant human APP in neuronal cultures using the SFV system and then metabolically labelled the cells for 4 h. The carboxyterminal fragments (CTFs) of APP were immunoprecipitated from cell lysates using an APP carboxyterminal-specific antibody and separated on 16% Tricine SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels for further analysis by a phosphorimager. We identified five species of CTFs (Fig 3A), as previously described (Hoey et al, 2009; Kimberly et al, 2005). We conclude that the two highest molecular weight bands are phosphorylated C99 and nonphosphorylated C99; the next faint band is phosphorylated C89; the fourth band is a mixture of non-phosphorylated C89 and phosphorylated C83; and the lowest molecular weight band is non-phosphorylated C83. In another group of experiments, we treated the immunoprecipitates with lambda protein phosphatase (LPP; Fig 3B) to remove the confounding phosphorylation, and this resulted, as predicted, in three species of CTFs, i.e. non-phosphorylated C99, C89, and C83. Quantification of phosphor images showed that C99, C89, and C83 accounted for 51.7, 24.9, and 23.4%, respectively, of the total of the three species (Fig 3C). These data indicate that β' -site cleavage is a major processing event that occurs with human APP in neuronal cultures. Interestingly, the E682K mutation (Fig 3A), when expressed in neuronal cultures, generated more C99 (both phosphorylated and non-phosphorylated), no detectable phosphorylated C89, and apparently less of the mixture of non-phosphorylated C89 and phosphorylated C83 compared to WT APP (Fig 3A). The level of the latter band was

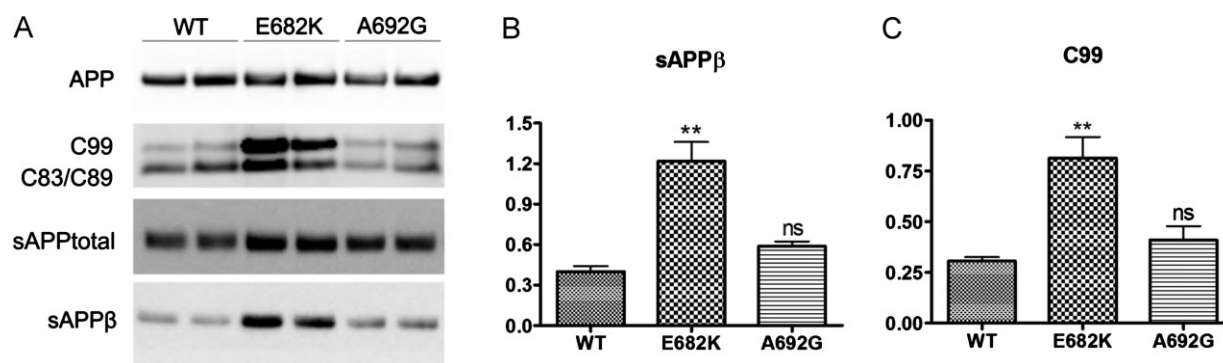


Figure 2. Effects of E682K mutation on APP processing in cultured neurons.

- A.** Primary cultured neurons were transduced with SFV expressing WT or mutant APP. Cell lysates were analysed by Western blotting using APP C-terminal antibody B63. Conditioned medium was analysed by Western blotting to determine secreted total sAPP (using 22C11 antibody) and sAPP β (using anti-sAPP β antibody).
- B, C.** Semi-quantification of sAPP β and C99 levels from Western blotting, data were normalized to APP levels (** $p < 0.01$; ns, statistically not significant; mean \pm SEM; $n = 4$).

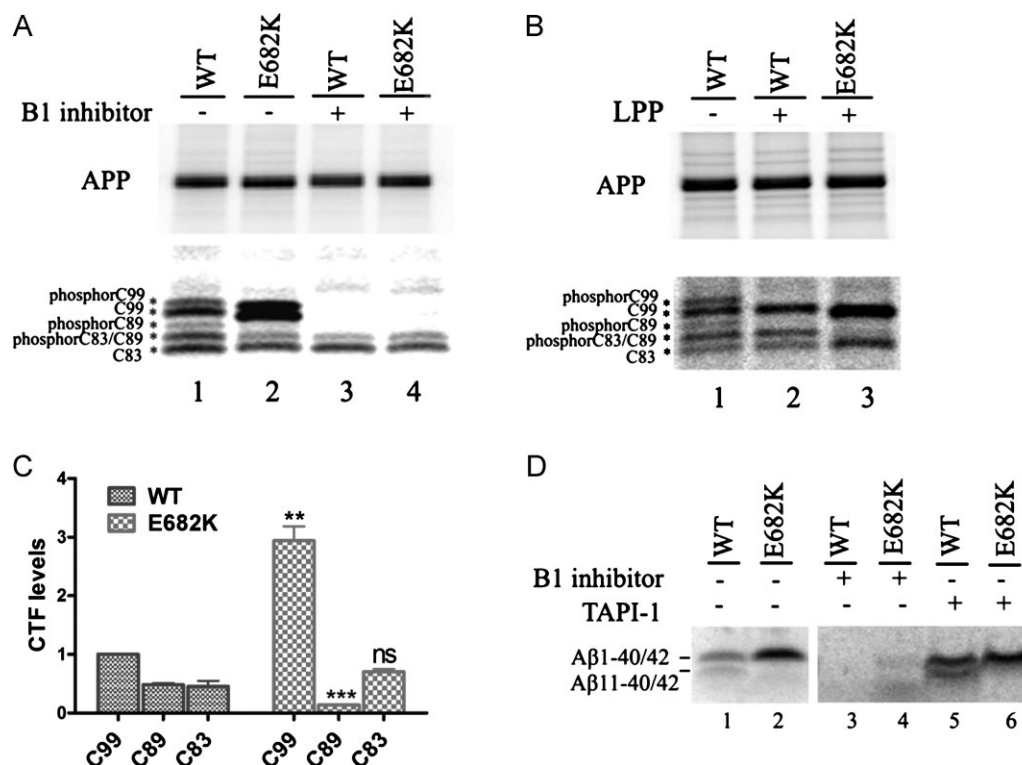


Figure 3. E682K mutation blocked β' -cleavage of human APP by BACE1 in cultured neurons. Primary cultured neurons were transduced with SFV expressing WT or E682K mutant APP and were metabolically labelled with [35 S] cysteine/methionine for 4 h.

- A.** Cell lysates were subjected to IP with antibody B63 specific to the C-terminus of APP. Eluates were separated on 16% Tricine gels and analysed by phosphorimager. Five species of CTFs were detected from neurons expressing WT APP (lane 1), including phosphorylated C99, C99, phosphorylated C89, C89, phosphorylated C83, and C83 (from high to low MW). Neurons expressing E682K APP (lane 2) eliminated the generation of phosphorylated C89 and C89. C89 can be measured by subtracting the density of the phosphorylated C83 band (lane 4) from the C89/phosphorylated C83 band (lane 2). In lanes 3 and 4, neurons were treated with BACE1 inhibitor, therefore, only phosphorylated and non-phosphorylated C83 is observed.
- B.** Immunoprecipitates from cell lysates were treated with LPP. Neurons expressing WT APP (lane 2) generated C99, C89, and C83, while neurons expressing E682K APP (lane 3) largely eliminated the generation of C89.
- C.** Quantification of radioactive bands of CTFs. Statistics were analysed by comparing each CTF species generated from E682K APP with that generated from WT APP (** $p < 0.01$; *** $p < 0.0001$; ns, statistically not significant; mean \pm SEM; $n = 4$).
- D.** Conditioned media were subjected to IP with JRFcA β 40/42 antibodies specific to the C-terminus of A β ₄₀ or A β ₄₂, eluates were resolved on 10% Bis-Tris gels and analysed by phosphorimager. Neurons expressing WT APP generated A β _{1-40/42} and A β _{11-40/42} species (lane 1), and production of both peptides was eliminated after treatment with BACE1 inhibitor (lane 3), but was unaffected by the treatment with α -secretase inhibitor TAPI-1 (lane 5). E682K mutation enhanced the generation of A β _{1-40/42} but eliminated the generation of A β _{11-40/42} (lanes 2 and 6).

equal to that of the band isolated from neuronal cultures treated with a highly selective BACE1 inhibitor compound 3 (Fig 3A), which is in accordance with our hypothesis that the E682K mutation blocked C89 generation. After treatment with LPP, this hypothesis was confirmed as only C99 and C83 were detected (Fig 3B).

We also immunoprecipitated A β related peptides from the conditioned medium using A β _{40/42} carboxyterminal specific monoclonal antibodies (mAbs). WT APP generated two major species (Fig 3D), and both peptides were eliminated after treatment with BACE1 inhibitor (Fig 3D), but remained after treatment with α -secretase inhibitor TAPI-1 (Fig 3D). We thus concluded that these two peptides were products from BACE1 cleavage, i.e. they are A β _{1-40/42} and A β _{11-40/42} species. In agreement with the results obtained from analyzing the CTF levels, the E682K mutation blocked the generation of the A β ₁₁₋

_{40/42} peptide while it enhanced generation of A β _{1-40/42} peptide (Fig 3D).

E682K mutation had little effects on α -secretase cleavage

The E682K mutation (Glu₁₁ to Lys) is located close to the α -secretase cleavage site Lys₁₇. Therefore, we decided to investigate whether this mutation affected α -secretase cleavage as well. The antibody 6E10, that we use to detect sAPP α , did not bind the E682K mutant APP in Western blotting (data not shown), however, the typical α -secretase generated C83 carboxyterminal fragment remained unchanged (Fig 3C), while total sAPP levels showed a slight increase probably due to the increase in sAPP β (Fig 2A), suggesting that this mutation had little effects on the α -site cleavage of APP. In contrast, the 'Flemish' A692G mutation appeared to slightly affect the generation of CTF α (Fig 2A), as published before (De Strooper et al, 1995; Haass et al, 1994).

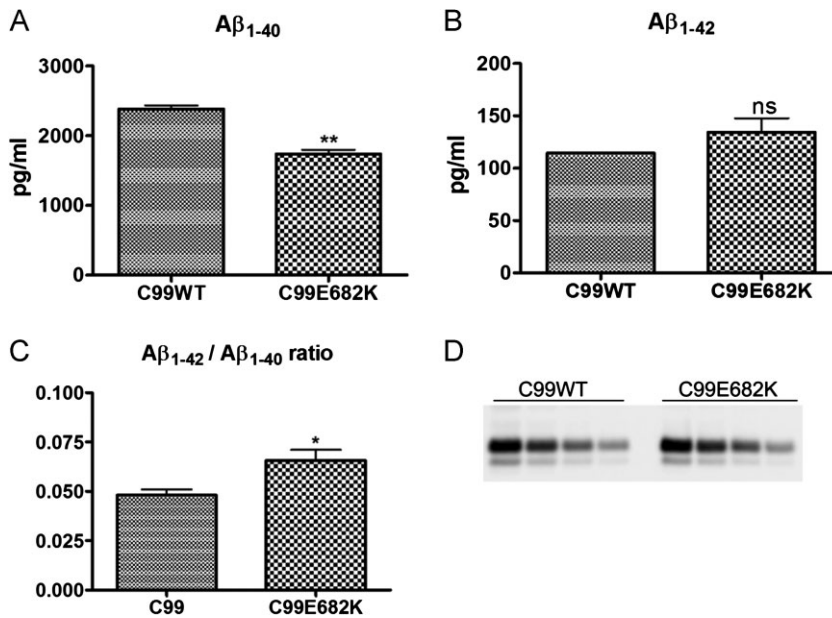


Figure 4. Effects of E682K mutant C99 on γ -secretase activity in cell-based assay.

A-C. CHO cells were transfected with WT or E682K mutant C99, conditioned media were analysed by A β ELISAs (** $p < 0.01$; * $p < 0.05$; ns, statistically not significant; mean \pm SEM; $n = 3$).

D. Western blotting analysis of C99 expression levels. Cell lysates containing either C99WT or C99E682K were loaded in a series of two times dilution.

E682K mutation modulated γ -secretase activity

One interesting observation is that the E682K mutation increased the A β_{1-42} /A β_{1-40} ratio in neuronal and CHO cell cultures. It is known that pathogenic mutations near the C-terminus of the A β sequence in APP can lead to increased generation of A β_{42} by modulating the active sites of γ -secretase. A recent report has shown that the 'Flemish' A692G mutation, which is located in the middle of A β , has unexpectedly, in addition to its slight inhibitory effect on α -secretase processing (De Strooper et al, 1995; Haass et al, 1994), a quite pronounced effect on γ -secretase activity (Tian et al, 2010), suggesting that the interaction between γ -secretase and C99 may also occur at sites that are remote from the actual cleavage site in C99. Therefore, we wondered whether the increase in the A β_{42} /A β_{40} ratio caused by the E682K mutation might be caused by an additional effect on the modulation of γ -secretase. We transiently transfected CHO cells with WT or E682K mutant C99. This APP fragment is the direct substrate of γ -secretase and its processing will, therefore, largely be determined by γ -secretase. The expression levels of both C99WT and C99E682K were investigated by Western blotting, no difference was observed. Conditioned media were analysed by A β ELISAs (Fig 4). The E682K mutation generated the same amounts of A β_{1-42} but significantly less A β_{1-40} , leading to an increased A β_{1-42} /A β_{1-40} ratio. These data suggested that the E682K mutation affects also to a certain extent the A β_{1-42} /A β_{1-40} ratio via modulation of γ -secretase.

Limited effects of E682K mutation on the aggregation kinetics and cytotoxicity of A β peptide

We studied the *in vitro* aggregation properties of synthetic A β peptide carrying the E682K mutation (E11K mutant A β). The aggregation kinetics of WT and mutant A β_{42} peptide were monitored by a Thioflavin T (ThT) fluorescence assay. Compared with WT A β_{42} peptide, E11K mutant A β_{42} showed a slight

increase in the initial aggregation rate during the first 3 h of the aggregation process (Fig 5A); however, the overall change in aggregation kinetics was small. Transmission electron microscopy (TEM) images further underlined that the aggregation process of E11K mutant A β_{42} was not significantly different from that of the WT A β_{42} peptide (Fig 5B). In parallel, we also analysed the cytotoxicity of E11K mutant and WT A β_{42} , no significant difference was detected (Fig S2 of Supporting Information).

DISCUSSION

This work describes the identification and characterization of a novel APP mutation E682K from a single case of early onset AD. The index patient is a Belgian female; she was diagnosed as probable AD at the age of 49 years. Neuropsychological evaluation, CSF biomarkers (A β_{42} , total-tau and phospho-tau), amyloid imaging (using ^{11}C -PIB PET) as well as magnetic resonance imaging of brain all confirmed the diagnosis of AD. The patient's father developed late onset AD, while the brother is an asymptomatic mutation carrier currently at the age of 53, and there is no extended familial information. This raised the interesting question whether the identified mutation is a benign polymorphism or should be considered a genuine genetic risk factor for disease. The issue of rare genetic variants contributing moderate risk to disease is hotly debated in genetic research, as it is very difficult to detect such mutations while they are likely to contribute significantly to the total genetic risk for disease (Singleton et al, 2010). We hypothesized that the APP E682K mutation could have variable penetrance, which has previously also been proposed for disease causing mutations in *PSEN2*, carriers of which display a strong variation in age of onset (Sherrington et al, 1996) and decided to use functional assays to test whether the identified mutation could be considered as pathogenic or not.

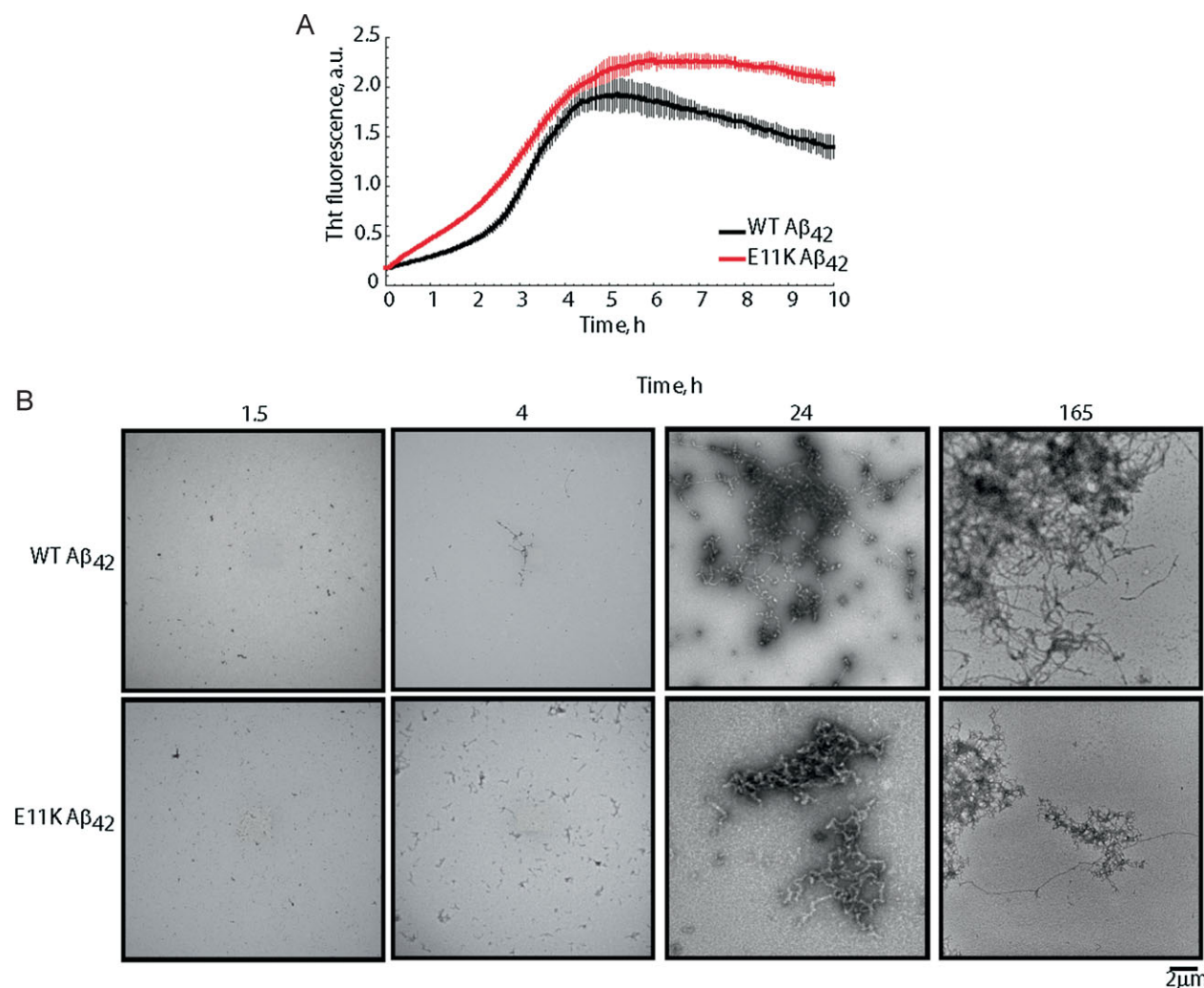


Figure 5. Biophysical determination of the aggregation kinetics of WT A β_{42} and mutant E11K A β_{42} .

- A.** ThT fluorescence of aggregating WT A β_{42} (black) and mutant A β_{42} E11K (red). Indicated are the error bars for each time point based on three measurements. The aggregation kinetics is not dramatically affected by the E11K mutation, apart from a small increase in the initial rate during the first 3 h of aggregation process.
- B.** TEM images further underline that the aggregation process of A β_{42} upon introduction of the E11K mutation is not significantly modified. After 1.5 h aggregates are observed which become larger upon further incubation.

Our analysis showed that this novel mutation increased full length A β release and also the A β_{1-42} /A β_{1-40} ratio to similar extents as found with a previously well characterized disease associated APP A692G or ‘Flemish’ mutation. While the observed effects on A β processing suggest the possibility that this novel mutation is pathogenic, the genetic support is inconclusive. According to the criteria discussed by Guerreiro and coworkers (Guerreiro et al, 2010), the APP E682K mutation was found in a single case of early onset AD. This mutation was absent from 940 healthy controls and affects A β generation. Although strictly spoken a second case with this mutation needs to be identified as independent confirmation (Guerreiro et al, 2010), we propose that the accumulated evidence is clearly in favour to classify this mutation as *probable pathogenic*. Further follow up of the brother of the index case could yield final

confirmation, but because of the study design, we are not allowed to contact the brother for further investigation. Therefore, we will have to wait for additional cases from other investigations in the future to classify this mutation as definitively pathogenic. Nevertheless, the functional analysis of this mutation provided an important and interesting novel insight in the biological significance of the β' -site processing of APP, which appeared quantitatively much more important for normal APP metabolism than previously thought.

Our data indicate indeed that processing at this site should be considered anti-amyloidogenic, counteracting partially the amyloidogenic β -secretase cleavage of APP. In agreement with this assumption, rodents, which tend to cleave their endogenous APP at the β' -site (De Strooper et al, 1995), do not spontaneously develop amyloid plaques. The functional significance of β' -site

cleavage of APP by BACE1 has indeed been debated since a long time. On the one hand, it was previously suggested that this cleavage is a minor part of physiological APP processing (Creemers et al, 2001). Our new study now clarifies that this is not the case and that about one-fourth of the major APP CTF species is generated by this pathway under our experimental conditions. Second, it was reported that N-terminal truncation of A β may enhance the aggregation properties as well as the cytotoxic effects of A β (Demeester et al, 2001) and that in sporadic AD cases, significant amounts of A $\beta_{11-40/42}$ species were found in the plaques in the brain (Huse et al, 2002; Liu et al, 2006). Therefore, β' -site cleavage was proposed to contribute to AD pathogenesis (Huse et al, 2002; Liu et al, 2006). However, our data argues for a protective role of β' -site cleavage, as blocking this cleavage disrupted the balance of APP processing by BACE1 leading to increased full length A β . The fact that the β' -site mutation E682K is associated with early onset AD speaks clearly in favour of this argument.

Of note, our data suggest additional effects of the E682K mutation on γ -secretase activity as well. APP carrying the E682K mutation increased significantly the A β_{1-42} /A β_{1-40} ratio in cell-based assays. This effect occurs at the γ -secretase level because expressing a C99 construct that bypasses the ectodomain shedding step needed for γ -secretase (Lichtenthaler et al, 1999) led to a similar increase in this ratio. It has been recently reported that a substrate inhibitory domain (ASID) located at the middle of A β (17–23) is modulating γ -secretase activity by binding to an allosteric site within the γ -secretase complex and that the 'Flemish' mutant C99 increased γ -secretase activity via disruption of this ASID (Tian et al, 2010). The E682K mutation is located at the A β_{11} site, which is not part of the previously delineated ASID, but had nevertheless a significant effect on A β_{1-40} levels and little effects on A β_{1-42} levels. We suggest the possibility that the interaction domain between γ -secretase and C99 is more extended than ASID and mutations such as the E682K mutation, as well as other similar substitutions, may be considered as new tools to investigate the possible allosteric modulation of γ -secretase by APP substrate.

The current data indicate that BACE1 cleaves APP at two distinct sites and that both are physiologically significant events in APP processing. So far, BACE1 is the most attractive drug target for AD treatments, and the evaluation of inhibitors under development for treatment of the disease focuses on the effect of blocking the β -site cleavage. This could be problematic because BACE1 inhibitors will also affect β' -site cleavage. Kinetic data showed that the BACE1 has a much higher enzymatic efficiency towards peptide containing the β -site than peptide containing the β' -site sequence (Yang et al, 2004), which raises a theoretical possibility that BACE1 inhibitors may have differential inhibition effects on the two distinct sites depending on the concentrations used. This could cause relative shifts from β' - to β -site cleavage, as observed with the current mutation. We thus propose that inhibitor drugs should be carefully monitored for their dose-effect on both BACE1 cleavage sites and to measure, therefore, the effects on A $\beta_{11-40/42}$ of any such drug when used in patients.

Finally it is likely that in the near future, increasing exon- and also exome-based sequencing efforts will increase the number of putative pathogenic mutations identified without further conclusive segregation-based evidence in a single family. Functional analysis of such mutations as demonstrated here will allow to determine the putative pathogenic nature of some of these mutations as more extensively discussed in (Guerreiro et al, 2010). Such functional study may also be crucial to fill part of the existing gap in genetic research with regard to the postulated low frequency, moderate risk genetic loci that are now very difficult to identify and were called the 'dark matter of disease risk' (Manolio et al, 2009; Singleton et al, 2010).

MATERIALS AND METHODS

Genetic analysis

Genomic DNA was extracted from whole blood by automated DNA extraction. Mutation analysis was performed by direct sequencing of exon 16 and 17 of APP. Twenty nanogram of genomic DNA was amplified in a polymerase chain reaction (PCR), and PCR products were purified using ExoSAP-IT[®] (USB Corporation, Cleveland, OH, USA) according to the manufacturer's protocol. Purified PCR products were sequenced in both directions (primer sequences available upon request), by fluorescent cycle sequencing using the 'Big Dye[®] Terminator v3.1 Cycle Sequencing' kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's recommendations. Labelled products were separated on an Applied Biosystems 3730 DNA analyser (Applied Biosystems). Sequence traces were analysed using NovoSNP (Weckx et al, 2005). The presence of g.278228G>A in exon 16 of APP in the index patient, and its absence from 940 control chromosomes was assessed using a pyrosequencing assay (primer sequences available upon request) on a PSQ[™] HS96A System (Biotage, Uppsala, Sweden) according to the manufacturer's protocol. Healthy control individuals were unrelated, age and gender matched and from the same geographical region.

Plasmids and site-directed mutagenesis

The plasmids pSG5-huAPP695, pSFV-huAPP695 (De Strooper et al, 1995) and pSG5-APPCC99-FLAG (Bentahir et al, 2006) have been described previously. Mutagenesis was performed using the Quick-Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Following primers were used to introduce the E682K mutation: 5'-CCGACATGACTCAGGATATAAAGTTCATCATCAAAAATTGGTG-3' (forward) and 5'-CACCAATTTTGTATGATGAACITTA-TATCCTGAGTCATGTCGG-3' (reverse).

Antibodies and compounds

Rabbit polyclonal antibody B63 raised against the C-terminus of APP has been described previously (Annaert et al, 2001). mAbs JRFcA β 40/28, JRFcA β 42/26 recognizing the C-terminus of A β species terminating at 40 or 42, respectively, and JRFABN/25 recognizing the N-terminal 1–7 amino acids of human A β were provided by Janssens Pharmaceutica. The following antibodies were purchased: mAb 22C11 recognizing the N-terminus of APP (Chemicon/Biognost); sAPP β polyclonal antibody recognizing the N-terminus to the β -secretase

cleavage site of APP (Covance). Compound TAPI-1 was purchased from Calbiochem; BACE1 inhibitor compound 3 was kindly provided by Merck Research Laboratories.

Amyloid imaging

Using a Siemens Biograph PET-CT scanner, ^{11}C -Pittsburgh Compound B images were acquired dynamically from 40 to 70 min following injection of 335.1 MBq. After realignment, PET frames were divided by the mean uptake in cerebellar cortex to obtain standard uptake ratio (SUVR) images. The index patient's SUVR image was nonlinearly warped to a PIB template in MNI space consisting of 13 AD patients previously rated as having increased uptake (Nelissen et al, 2007). Volume-of-interest (VOI) masks were derived from the Automatic Anatomical Labelling (AAL) atlas, whereby, a composite cortical VOI was defined as the union of parietal, lateral temporal and frontal areas excluding primary sensory motor cortex.

Cell culture and transfection

CHO cells were maintained at 37°C in a humidified, 5% CO_2 controlled atmosphere in DMEM (Gibco) supplemented with 10% foetal bovine serum (FBS). Transfections were performed using Fugene (Invitrogen) according to the manufacturer's instructions. When 80% confluence was reached, cells were transiently transfected with APP or C99 cDNA constructs. After 24 h, media were replaced with DMEM supplemented with 5% FBS and conditioned for 16–24 h and then collected for A β ELISA assays in the presence of complete protease inhibitor cocktail (Roche). Conditioned media were also analysed by Western blot for the detection of sAPP, while cell lysates were analysed by Western blot for the detection of APP CTFs.

Neuronal cultures and Semliki Forest virus transfection

Primary cortical neurons were derived from E14 embryos from C57BL/6 mice, the preparation procedures have been described previously (Annaert et al, 1999). Briefly, the brains of E14 embryos were dissected in HBSS medium (Gibco), trypsinized and plated on 6-cm dishes (Nunc) precoated with poly-L-lysine (Sigma–Aldrich). Cultures were maintained in neurobasal medium (Gibco) supplemented with B27 (GibcoBRL). A 5 μM cytosine arabinoside was added to prevent glial cell proliferation. Semliki Forest viruses (SFV) were produced as described (Annaert et al, 1999). Three days cultured neurons were transduced with SFV expressing WT or mutant human APP695. After 1 h, media were replaced with normal culture media and left for 2 h. Media were refreshed and conditioned for ~6 h. Conditioned media were analysed by A β ELISA or Western blot for the detection of sAPP, while cell lysates were analysed by Western blotting for the detection of APP CTFs.

Metabolic labelling and Immunoprecipitation

Neuronal cultures were transduced with SFV for 1 h and left in normal culture medium for 2 h as described above. Media were then replaced with methionine-free MEM (Gibco BRL) containing 100 $\mu\text{Ci/ml}$ [^{35}S] methionine (ICN Biomedicals). After 4 h metabolic labelling, supernatants were collected, cells were washed and lysed in DIP buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) supplemented with complete protease inhibitors. Supernatants were immunoprecipitated using a combination of JRFcA β 40 and JRFcA β 42 antibodies and incubated with protein G-Sepharose at 4°C overnight. The immunoprecipitants were resolved

on 10% NuPAGE gels (Novex; Invitrogen) running with MES buffer. Cell extracts were immunoprecipitated using APP C-terminal antibody B63. The immunoprecipitants were resolved on 16% Tricine gels (Novex; Invitrogen) running with Tricine–SDS buffer (Novex; Invitrogen). The intensity of the radioactive bands was quantified using a phosphorimager (Molecular Dynamics, Suonyvale, CA) and Aida Image Analyzer v.4.22 software.

A β ELISA

The concentration of A β in conditioned medium was measured by end-specific ELISAs. JRFcA β 40/28 or JRFcA β 42/26 was used as capturing antibodies for A β species terminating at 40 or 42, respectively. HRP-conjugated JRFABN/25 recognizing the N-terminal 1–7 amino acids of human A β was used as detecting antibody for full length A β . The affinity of JRFABN/25 for E11K mutant A β peptide was not different from its affinity for WT A β peptide (Fig S3 of Supporting Information). Plates were coated with 60 μl capturing antibodies at 2 $\mu\text{g/ml}$ at 4°C overnight in coating buffer (10 mM Tris–HCl, 10 mM NaCl, 10 mM NaN_3 , pH 8.5). The next day, plates were rinsed once in phosphate buffer saline (PBS) and blocked with blocking buffer (0.1% casein in PBS, pH7.4) either at 4°C overnight or at room temperature for 4 h. Plates were then rinsed twice with PBS. Thirty microlitre of detecting antibody diluted in blocking buffer was first added to each well, followed by adding 30 μl conditioned medium diluted in blocking buffer. Plates were incubated at 4°C overnight, and rinsed and developed the next day using 60 μl mixture of 3,3',5,5'-tetramethylbenzidine (TMB) solution. The reaction was stopped by addition of 60 μl of 2 N H_2SO_4 . Plates were read immediately at 450nm.

Preparation of A β peptide

A β peptide (JPT Peptide Technologies GmbH) was dissolved at a concentration 1 mg/ml in 1,1,1,3,3,3-hexafluor-2-propanol (HFIP 99%, Aldrich Cat.# 10,522-8). HFIP was evaporated using a gentle stream of argon gas and the peptide film was resolved using dimethyl sulphoxide, (DMSO Sigma Cat. #D4540) at a final concentration of 1 mg/ml. The peptide was separated from DMSO with a 5 ml HiTrapTM Desalting column (GE Healthcare, Sweden). Complete removal of DMSO was confirmed by Fourier transform infrared spectroscopy (FTIR): DMSO provides spectral maxima at 1011 and 951 cm^{-1} . The peptide was eluted into a 50 mM Tris, 1 mM EDTA buffer, pH 7.5 and the peptide concentration was measured using Bradford assay. The samples were kept on ice until experiments started, with a maximum lag time of 20 min.

Thioflavin T fluorescence

A β protein concentrations were normalized to 25 μM by further dilution using 50 mM Tris, 1 mM EDTA containing buffer and a final concentration of 12 μM ThT was added in a Greiner 96-well plate. The fibrillation kinetics were followed *in situ* using a Fluostar OPTIMA fluorescence plate reader at an excitation wavelength of 440 nm and an emission wavelength of 480 nm. Readings were recorded in triplicate every 10 min for a period of 10 h.

Transmission electron microscopy

Aliquots (5 μl) of the A β preparation were adsorbed to carbon-coated FormVar film on 400-mesh copper grids (Plano GmbH, Germany) for

The paper explained

PROBLEM:

Direct genomic sequencing of *APP* exons 16 and 17 in a group of Belgian early onset Alzheimer's disease (AD) patients ($n = 543$) identified a novel mutation in exon 16 (g.278228 G>A, c.2044G>A), predicting a glutamine-to-lysine substitution at codon 682 (p. E682K). This mutation was absent from healthy control group ($n = 940$). The index patient was diagnosed as probable AD at the age of 49 years. Neuropsychological evaluation, CSF biomarkers (A β 42, total-tau and phospho-tau), amyloid imaging (using 11C-PIB PET) as well as magnetic resonance imaging of brain all confirmed the diagnosis of AD. The patient's father developed late onset AD, while the brother is an asymptomatic mutation carrier currently at the age of 53, and there is no extended familial information. Genetic information is nonconclusive in determination of the pathogenicity of the identified mutation. This work explored the possible pathogenic nature of the novel APP mutation by functional analysis.

RESULTS:

In both neuronal and non-neuronal cell cultures, APP E682K mutation caused a two- to three-fold increase in A β ₁₋₄₀ and A β ₁₋₄₂ levels. The E682K mutation is located at the alternative β' -secretase cleavage site on APP, the quantitative significance of which remained elusive. This work shows that the β' -processing of human APP is a major event in neuronal cultures, whereas, the E682K mutation blocked the β' -site cleavage and shifted APP processing to the β -site, thus enhanced the generation of full length A β as well as other β -cleavage products from APP.

Interestingly, the E682K mutation also led to a significant increase in the ratio of A β ₁₋₄₂ to A β ₁₋₄₀. Our data suggest that this is due to modulation of γ -secretase, as introducing the mutation to C99, a direct substrate for γ -secretase, significantly increased the ratio of A β ₁₋₄₂ to A β ₁₋₄₀ in cell cultures.

The alterations on A β generation caused by E682K mutation are quantitatively similar to those caused by the previously reported disease causing 'Flemish' A692G mutation. Functional analysis strongly supports the possible pathogenic nature of the novel mutation.

IMPACT:

This work first demonstrates that β' -site cleavage of human APP is a major event in neurons and plays a 'protective' role in APP metabolism, while disruption of this cleavage by mutation or other effects will enhance the amyloidogenic processing of APP and may consequentially lead to AD.

Our work shows how a combination of clinical and cell biological methods works to determine the possible pathogenicity of novel mutations identified in single families when segregation-based evidence is not available.

This work is important from a drug development perspective as well, adding a potentially important layer of complexity to the development of the β -secretase (BACE1) inhibitors in the clinic. We propose that β -secretase inhibitors should be monitored for their effects on both the β - and the β' -site. Inhibitors at certain concentration blocking the β' -site and shifting the cleavage of APP to β -site could be problematic.

1 min. The grids were blotted, washed twice in droplets of Milli-Q water, and stained with 1% w/v uranyl acetate. Samples were studied with a JEOL JEM-1400 microscope at 80 kV.

Cell viability assay

Mouse primary hippocampal neurons were grown in Neurobasal medium (Invitrogen) for 1 week, and then were treated with different concentrations of A β 42 (WT or mutant) pre-aggregated for 1.5 h. After 72 h treatment, 10 μ l Cell-Titer-Blue dye (Promega) was added to 200 μ l of the growth culture medium on the cells. After 3 h, the fluorescence intensity of the samples was measured at an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

Statistical analyses

Statistical significance was determined by the Student's *t*-test.

Author contributions

LZ analysed the effects of APP mutants in A β generation and wrote the manuscript. NB and KS performed the genetic analysis. MM suggested experiments and contributed to A β

ELISA measurements. KVL, PVD, DD and FVL analysed patient's brain image and blood samples. AV, KB and IB characterized the aggregation property and cytotoxicity of mutant A β peptide *in vitro*. CVB and RV identified the AD case and the APP mutation. BDS designed the study and wrote the manuscript.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

For more information

Online Mendelian Inheritance in Man (OMIM)
APP

<http://www.ncbi.nlm.nih.gov/omim/104760>

BACE1

<http://www.ncbi.nlm.nih.gov/omim/604252>

Alzheimer Research Forum:

<http://www.alzforum.org/>

Alzheimer's Disease Mutation Database:

<http://www.molgen.ua.ac.be/ADMutations/>

Bart De Strooper's laboratory:

<http://med.kuleuven.be/cme/subpage.html?section=laboratories&subsection=laboratory-for-the-research-of-neurodegenerative-diseases>

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